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# Biomonitoring ambient air quality using leaf characteristics of trees

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# Summary

Traffic, industry, agriculture and the burning of fossil fuels have brought a large amount of anthropogenic air pollutants, such as sulphur dioxide ($SO_2$), particulate matter (PM), nitrogen dioxide ($NO_2$), carbon dioxide ($CO_2$) and ammonia ($NH_3$) into the atmosphere. To understand the - short or long-term - impact of these chemical compounds on ecosystems, biomonitoring is applied as a powerful, cost effective, user friendly tool for filling the gap between doses of, and responses to air quality. Plants are more frequently used as biomonitor than humans and animals because plants have a broad geographical distribution, are easy to gather and reflect better the local conditions, since they are more sensitive in terms of physiological reaction to the common air pollutants. Most of the biomonitoring studies do not reflect real-life situations since they investigate the influence of only one single air pollutant on plants, at (extremely) high concentrations and under laboratory conditions. The general aim of this thesis was to gain insight into the impact of ambient air quality on leaf characteristics of trees under field conditions, in order to investigate the potential of these leaf characteristics for (active and passive) biomonitoring purposes. In addition, species-dependent responses to ambient air quality as well as the influence of exposure time to air pollution on the response of white willow was investigated.

The passive biomonitoring with common oak (*Quercus robur*) was not able to detect differences in $NH_3$ concentration, probably due to many confounding variables that mask the possible effect of $NH_3$. Therefore, preference is given to monitor the ambient air quality by using the active biomonitoring approach. Our first active biomonitoring study, performed in a rural and an urban area, indicated that white willow (*Salix alba* L.) is a potentially good species to monitor ambient air quality, since it is a fast growing species and it allows the use of stem cuttings, giving the advantage that phenotypic variation is likely to be a reflection of the environment experienced rather than genotypic differences. Willow leaves produced more and

smaller stomata in the urban area, compared to the rural area, to optimize stomatal closure efficiency and to limit gas diffusion by increasing stomatal resistance ($R_S$). To accommodate the water deficiency and herbivory problems encountered in this first study, a semi-automatic, capillarity-based, water supply-system was developed and copper tape was used to avoid snail herbivory in the following biomonitoring studies. In addition, we planted white willow in the near vicinity of air quality monitoring stations of the Flemish Environmental Agency, Institut Scientifique de Service Public and Brussels Institute for Management of the Environment to correlate changes in leaf characteristics with ambient air quality data.

Each leaf characteristic showed its own tolerance against ambient air pollution. Leaf area fluctuating asymmetry, stomatal density, leaf wettability, maximum photochemical efficiency of photosystem II, ascorbate, glutathione and flavonoid content, superoxide dismutase, peroxidase and ascorbate peroxidase activity and $\delta^{15}N$ of white willow were not linked with ambient air pollution, while specific leaf area (SLA), $R_S$, malondialdehyde content (MDA), total antioxidant capacity (FRAP) and polyphenol (POLY) content increased and $\delta^{13}C$ decreased with increasing $NO_2$ concentration. These adaptations indicate a toxic effect of $NO_2$: (i) increased SLA to induce the inhibition of photosynthesis, (ii) increased $R_S$ to minimize the uptake of $NO_2$, (iii) increased MDA as a consequence of the peroxidation of poly-unsaturated fatty acids and (iv) decreased $\delta^{13}C$ as a consequence of changed stomatal conductance and/or biochemical characteristics negatively affecting photosynthesis. Two-year exposure of willow to the ambient air quality hardly influenced the response of the measured leaf characteristics. Only $R_S$, which was correlated to meteorological conditions in the first in-leaf season, was affected by $NO_2$ in the second in-leaf season due to the formation of smaller stomata. Besides air pollution, also shade influenced the leaf characteristics through the production of thinner, more wettable leaves with a lower ascorbate and glutathione content. To avoid the effect of shade, we suggest sampling sites with a similar degree of shadow, by taking leaves from unshaded positions, and/or measuring leaf characteristics that are less sensitive to shadow, such as MDA and POLY.

The response of leaf characteristics to ambient air quality is species-dependent, as shown by the active biomonitoring study with white willow, northern red oak (*Quercus rubra* L.) and Scots pine (*Pinus sylvestris* L.). The SLA and maximum photochemical efficiency of photosystem II ($F_v/F_m$) of pine increased and leaf area fluctuating asymmetry (FAA) and $F_v/F_m$ of oak

increased with an increasing atmospheric $NO_2$ and decreasing $O_3$ concentration. We concluded that biochemical measurements are needed to find out whether $O_3$ is toxic or whether $NO_2$ has a fertilizing effect on oak and pine. However, based on practical considerations (e.g., ease of transport and planting) and the adaptations of the leaf characteristics of willow to $NO_2$, willow seemed to have more potential for biomonitoring ambient air quality compared to oak and pine.

In conclusion, biomonitoring ambient air quality under field conditions is a difficult task due to (i) the different responses of species, leaf characteristics and pollutant-dependent responses and (ii) the complex interaction of several other environmental factors (e.g., air temperature, relative air humidity, wind) with air pollution in an unknown way and modifying the response of plants to air pollution. This leads to an unavoidable variability in the responses from leaves of the same plant and between plants at the same site. In addition, environmental factors can influence leaf characteristics in the same way air pollution does, making it difficult to properly interpret the obtained results. Passive biomonitoring has the additional disadvantage of genetic pollution besides soil and age effects, which lead to inability to obtain information about the ambient $NH_3$ concentration by using common oak as a passive biomonitor.

# Samenvatting

Verkeer, industrie, landbouw en het verbranden van fossiele brandstoffen brengen een grote hoeveelheid antropogene luchtpolluent, zoals $SO_2$, PM, $NO_2$, $CO_2$, $NH_3$ en zware metalen. Om de effecten op korte en/of lange termijn van al deze chemische componenten op ecosystemen te begrijpen, wordt biomonitoring toegepast als een kosteneffectieve, gebruiksvriendelijke methode om dose-response relaties op te stellen. Voornamelijk worden planten gebruikt in dergelijke studies, aangezien planten een bredere geografische distributie kennen, eenvoudiger te verzamelen zijn en beter de lokale condities reflecteren ten opzichte van mensen en dieren. Een groot deel van deze biomonitoringstudies onderzoeken echter de invloed van een hoge concentratie van één polluent op planten en onder laboratoriumomstandigheden. Het doel van het doctoraatsonderzoek was dan ook om inzicht te verkrijgen in de impact van de heersende luchtkwaliteit op bladkarakteristieken van bomen, om zo het potentieel van deze bladkarakteristieken te onderzoeken voor (actieve en passieve) biomonitoring doeleinden.

De passieve biomonitoring met zomereik (*Quercus robur*) was niet geschikt om verschillen in $NH_3$ concentraties op te meten, waarschijnlijk omwille van de aanwezigheid van parameters die het werkelijke effect van $NH_3$ maskeren. Daarom wordt de voorkeur gegeven aan actieve biomonitoring om de heersende luchtkwaliteit te monitoren. De eerste actieve biomonitoring werd zowel uitgevoerd in een landelijk als in een stedelijk gebied. Deze studie toonde aan dat schietwilg een goede soort is om de heersende luchtkwaliteit te monitoren, aangezien wilg een snelgroeiende soort is en er stekken kunnen gebruikt worden om genetische pollutie te vermijden. Bovendien toonde deze studie aan dat wilg meer en kleinere stomata vormt in stedelijke gebieden dan in landelijke gebieden, om de sluitefficiëntie te optimaliseren en de gasdiffusie te limiteren. Er traden echter problemen op i.v.m. watertekort en vraat, waardoor in een tweede biomonitoringstudie een semi-automatisch watertoevoersysteem werd ontwikkeld en koperband werd gebruikt om slakkenvraat te vermijden. Wilg werd vervolgens ook

geplant in de nabijheid van luchtkwaliteitmonitoringstations om de aanpassing van bladkarakteristieken te kunnen linken aan de heersende luchtkwaliteit.

Elk bladkarakteristiek vertoonde zijn eigen tolerantie ten opzichte van deze heersende luchkwaliteit. Fluctuerende asymmetry van een bladoppervlak, stomatale densiteit, bladhydrofobiciteit, de maximale fotochemische efficiëntie van fotosysteem II, ascorbaat-, glutathion- en flavonoid-gehalte, superoxide dismutase-, peroxidase- en ascorbaat peroxidase-activiteit en $\delta^{15}$N waren niet gecorreleerd met de heersende luchtkwaliteit, terwijl specifieke bladoppervlakte (SLA), stomatale weerstand ($R_S$), malondialdehyde (MDA), totaal antioxidantcapaciteit (FRAP) en polyfenolen (POLY) gehalte toenamen en $\delta^{13}$C afnam met een toenemende atmosferische $NO_2$ concentratie. Al deze aanpassingen tonen een toxisch effect van $NO_2$ aan: (i) SLA neemt toe om fotosynthetische inhibitie te compenseren, (ii) $R_S$ neemt toe om de opname van $NO_2$ te reduceren, (iii) MDA neemt toe als een gevolg van de oxidatie van poly-onverzadigde vetzuren en (iv) $\delta^{13}$C nam af omwille van een gewijzigde stomatale geleidbaarheid en/of biochemische kenmerken die de fotosynthese negatief benvloeden. Bovendien heeft een tweejarige blootstelling van wilg aan de heersende kwaliteit nauwelijks een invloed op de respons van de bladkenmerken. Enkel $R_S$, welke gecorreleerd was met de meteorologische condities gedurende het eerste onderzoeksjaar, werd negatief beïnvloed na twee jaar blootstelling aan $NO_2$ door de vorming van kleinere stomata. Naast luchtkwaliteit heeft ook schaduw een significante invloed op de opgemeten bladkenmerken. Bladeren gevormd in de schaduw waren dunner, minder hydrofoob en bevatten een hoger ascorbaat- en glutathion-gehalte. Om de invloed van schaduw zoveel mogelijk te reduceren, wordt aangeraden om (i) locaties te selecteren met een vergelijkbare hoeveelheid schaduw, (ii) bladeren te bemonsteren van een niet-schaduwrijke positie en/of (iii) bladkenmerken op te meten die minder gevoelig zijn voor schaduw zoals MDA en POLY.

De respons van bladkenmerken op de heersende luchtkwaliteit is ook soortafhankelijk, zoals aangetoond door de actieve biomonitoring met schietwilg, Amerikaanse eik en grove den. SLA en $F_v/F_m$ van den nam toe en FAA en $F_v/F_m$ van eik nam toe met een toenemende atmosferische $NO_2$ concentratie en vice versa. Biochemische metingen zijn noodzakelijk om na te gaan of $O_3$ een toxische invloed of $NO_2$ een bemestende invloed uitoefende op Amerikaanse eik en grove den. Gebaseerd op de praktische bemerkingen (bv. het gemak bij transport en planten) en de gevoeligheid van

de bladkenmerken van schietwilg voor $NO_2$, besluiten we dat schietwilg meer potentieel heeft om te gebruiken als actieve biomonitor in vergelijking met Amerikaanse eik en grove den.

In het algemeen is biomonitoring van de heersende luchtkwaliteit aan de hand van bladkarakteristieken is een moeilijke taak omdat (i) soort-, blad- en polluent-afhankelijk responsen aanwezig zijn en (ii) verschillende omgevingsfactoren op een ongekende manier interageren met atmosferische polluenten waardoor de respons van planten op luchtkwaliteit wijzigt. Deze omgevingsfactoren geven dus aanleiding tot een onvermijdbare variabiliteit in respons tussen planten van eenzelfde site en tussen bladeren van eenzelfde plant. Bovendien kunnen deze factoren ook eenzelfde invloed uitoefenen op de bladkarakteristieken als de heersende luchtkwaliteit, waardoor de bekomen resultaten enorm moeilijk te interpreteren zijn. Bovendien heeft passieve biomonitoring ook nog het nadeel dat taxonomische identificatie moeilijk is, waardoor genetische variabiliteit ontstaat, en dat er mogelijks verschillen in bodemkenmerken en/of leeftijd voorkomen.

# List of Abbreviations and Symbols

## A

| | |
|---|---|
| AOT40 | accumulated exposure over a treshhold of 40 ppb ozone (μg $m^{-3}$ hours) |
| APX | ascorbate peroxidase enzyme (μmol ASC $mg^{-1}$ protein $min^{-1}$) |
| AS | antisymmetry |
| ASC | reduced ascorbate (μmol $g^{-1}$ FW) |

## C

| | |
|---|---|
| C | carbon |
| CA | drop contact angle (°) |

## D

| | |
|---|---|
| DA | directional asymmetry |

## F

| | |
|---|---|
| FA | fluctuating asymmetry (-) |
| FAA | leaf area fluctuating asymmetry (-) |

| | |
|---|---|
| FLA | flavonoid content (mg quercetin $g^{-1}$ FW) |
| FRAP | total antioxidant capacity (µmol trolox $g^{-1}$ FW) |
| $F_v/F_m$ | maximum photochemical efficiency of photosystem II (-) |

## G

| | |
|---|---|
| GSH | reduced glutathione (µmol $g^{-1}$ FW) |

## L

| | |
|---|---|
| LA | leaf area of the left lamina side ($cm^2$) |
| L | stomatal length (µm) |

## M

| | |
|---|---|
| MDA | malondialdehyde (nmol $g^{-1}$ FW) |
| MLA | mean leaf area ($cm^2$) |
| MLB | mean leaf biomass (g) |

## N

| | |
|---|---|
| N | nitrogen |
| $NH_3$ | ammonia |
| NO | nitrogen monoxide |
| $NO_2$ | nitrogen dioxide |
| $NO_x$ | nitrogen oxide |

## O

| | |
|---|---|
| $O_3$ | ozone |

## P

| | |
|---|---|
| PCA1 | first principal component axis, site specific value for air quality |
| PCA2 | second principal component axis |
| PI | performance index (-) |
| PLA | projected leaf area ($cm^2$) |
| PM | particulate matter |
| $PM_{10}$ | particulate matter with an aerodynamic diameter of 10$\mu$m |
| $PM_{2.5}$ | particulate matter with an aerodynamic diameter of 2.5$\mu$m |
| POLY | polyphenol content (mg gallic acid $g^{-1}$ FW) |
| POX | peroxidase enzyme ($\mu$mol pyrrogalloline $mg^{-1}$ protein $min^{-1}$) |

## R

| | |
|---|---|
| RA | leaf area of the right lamina side ($cm^2$) |
| RCC | relative chlorophyll content (-) |
| RH | relative air humidity (%) |
| ROS | reactive oxygen species |
| $R_S$ | theoretical minimal stomatal resistance (s $m^{-1}$) |

## S

| | |
|---|---|
| SB | shoot biomass (g) |

| | |
|---|---|
| SD | stomatal density ($mm^{-2}$) |
| SLA | specific leaf area ($cm^2\ g^{-1}$) |
| $SO_2$ | sulfur dioxide |
| SOD | superoxide dismutase enzyme (unit SOD $mg^{-1}$ protein $min^{-1}$) |
| SPS | stomatal pore surface ($\mu m^2$) |

## T

| | |
|---|---|
| T | air temperature (°C) |

## V

| | |
|---|---|
| VOC | volatile organic compound |
| VPD | vapor pressure deficit (Pa) |

## W

| | |
|---|---|
| W | stomatal width ($\mu m$) |

# Symbols

$\delta^{13}C$ stable carbon isotope ratio (‰)
$\delta^{15}N$ stable nitrogen isotope ratio (‰)

# 1 Introduction

## 1.1 Air pollution

*A clean air supply is essential for our own health and that of the environment. Since the industrial revolution, the quality of the air we breathe has deteriorated considerably - mainly as a result of human activities. The rising industrial activity and energy production, the burning of fossil fuels and the dramatic rise in traffic on our roads all contribute to air pollution in our towns and cities (ec.europa.eu).*

### 1.1.1 Definitions

Defining air pollution is not a simple task, which leads to numerous definitions. Daly and Zannetti (2007) define air pollution as 'any substance emitted into the air from an anthropogenic, biogenic or geogenic source, that is either not part of the natural atmosphere or is present in higher concentrations than in the natural atmosphere, and may cause short-term or long-term adverse effects'. The World Health Organization defines air pollution as 'a contamination of the indoor or outdoor environment by any chemical, physical or biological agent that modifies the natural characteristics of the atmosphere' (www.who.org), while according to the United Nations Economic Commission for Europe, air pollution means the introduction by man, directly or indirectly, of substances into the ambient air resulting in

deleterious effects of such a nature as to endanger human health, harm living resources, ecosystems, material property and impair or interfere with amenities and other legitimate uses of the environment (www.unece.org). In this thesis air pollution is defined as 'an increased concentration of atmospheric chemicals (i.e., air pollutants)'; synergistic (positive) and antagonistic (negative) interactions between these air pollutants results in ambient air quality.

### 1.1.2 Sources of air pollution

Pollutants are classified as either primary or secondary pollutants. A primary pollutant is one that is emitted into the atmosphere directly from the source of the pollutant and retains the same chemical form, such as sulfur dioxide ($SO_2$), nitrogen dioxide ($NO_2$), carbon dioxide ($CO_2$) and ammonia ($NH_3$). Sources of air pollution can be divided into biogenic sources (e.g., trees emit volatile organic compounds (VOC)), geogenic sources (e.g., radionuclides from radioactive soil minerals, volcanoes emit particulate matter (PM)) and human-generated or anthropogenic sources, which are further divided into mobile and stationary sources (www.epa.gov). Mobile sources of anthropogenic air pollution include most forms of transportation such as automobiles, trucks and airplanes, while stationary sources or point sources of anthropogenic air pollution consist of non-moving sources such as power plants, oil refineries and other industrial facilities (www.epa.gov). Figure 1.1 gives a detailed description of the main sources of air pollutants, such as $SO_2$, nitrogen oxides ($NO_x$) and $PM_{10}$ (particles with an aerodynamic diameter smaller than 10 μm) in northern Belgium.

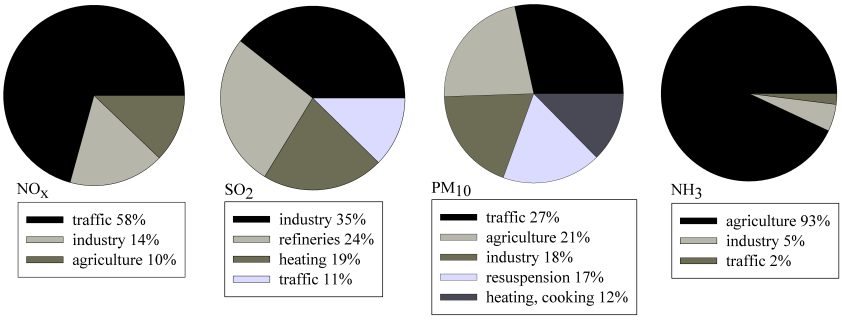


Figure 1.1: The main sources of the primary pollutants, $SO_2$, $NO_x$, $PM_{10}$ and $NH_3$ for Flanders, northern Belgium, in 2010 (www.vmm.be)

Ozone ($O_3$), a secondary pollutant, is formed by photochemical reactions

of its precursors like $NO_2$, hydrocarbons and VOC. These photochemical reactions are favoured by high temperatures and high light intensities with relatively slow winds (Wahid et al. 2001). Field studies have revealed that $O_3$ generated at urban-industrial locations may travel 50-1000 km from the point of origin in the direction of the prevailing level of atmospheric turbulence and penetrate deep into rural areas (Gregg et al. 2003, Emberson 2009). As a consequence, $O_3$ concentrations tend to be higher in suburban and rural locations compared to urban locations.

### 1.1.3 Air quality limit values and policy measures

#### 1.1.3.1 Europe and United States

The United States air quality management regimes started with the Air Pollution Control Act of 1955, which provided funds to investigate health and welfare effects of air pollution. Based on these scientific studies, air quality criteria could be defined, leading to the US Clean Air Act of 1963. The Clean Air Act was a milestone for the implementation of national air pollution laws and regulations all over the world.

The European Union air quality management regimes started in 1980 with Directive 80/779/EEC, which set air quality limit values and guide values for $SO_2$ and suspended particulates. Later Directives set limit values for lead, $NO_2$ and $O_3$. The 1996 Air Quality Framework Directive (96/62/EC) and its daughter directives formed the basis for the ambient air quality policy in the European Union. In 2008, most of the legislation, except for the $4^{th}$ daughter directive, has been merged into Directive 2008/50/EC. The four daughter directives set limit values for several air pollutants for prolonged exposure to low concentrations and for short-term exposure to high concentrations of air pollutants, in order to protect human health and ecosystems (ec.europa.be, Table 1.1). For $NH_3$, the critical level concept of 8 $\mu g\ NH_3\ m^{-3}$ for environmental protection has been set (Krupa 2003, Pitcairn et al. 2003). For $PM_{2.5}$, Directive 2008/50/EC recognizes that no threshold has been identified below which this pollutant would not pose a risk. It should be noted that the EU health- and vegetation-based limit values in Table 1.1 generally exceed those of the World Health Organization.

To ensure that the air quality limit values are met, various national strategies were developed to reduce emissions from large combustion plants (industrial boilers burning fuel to generate electricity and/or heat) and other major industrial installations, as well as from road vehicles and other mobile sources such as ships (ec.europa.eu). The $NH_3$ concentration, mainly

emitted by intensive livestock (agriculture, Fig. 1.1), is reduced by several abatement measures, such as dietary manipulation, storage, land application and fertilizer substitution measures (Cowell and Apsimon 1998, Olivier et al. 1998).

Table 1.1: Human health- and vegetation*-based limit values for a number of air pollutants ($SO_2$, $NO_2$, $PM_{10}$ and $O_3$), AOT40 (the accumulated exposure over a treshhold of 40 ppb during the months May - July, $\mu g\ m^{-3}$ hours) and the number of permitted exceedances, developed by the European Union (ec.europa.be); n/a: not applicable

| Pollutant | Concentration ($\mu g\ m^{-3}$) | Averaging period | Permitted exceedances/yr |
|---|---|---|---|
| $SO_2$ | 350 | 1 hour | 24 |
| | 125 | 24 hours | 3 |
| | 20* | 1 year | n/a |
| $NO_2$ | 200 | 1 hour | 18 |
| | 40 | 1 year | n/a |
| | 30* | 1 year | n/a |
| $PM_{10}$ | 50 | 24 hours | 35 |
| | 40 | 1 year | n/a |
| $O_3$ | 120 | maximum of daily 8 hour mean | 25 days over 3 years |
| AOT40 | 18 000* | 5 year | n/a |

Air pollution can also be transported over very long distances by wind, which means that air pollution is not only a national, but also an international issue. Since 1979, cooperation within the UNECE region has been driven by the Convention on Long-range Transboundary Air Pollution, which has contributed to the development of international environmental laws and created essential frameworks for controlling and reducing the damage to human health and ecosystems, caused by transboundary air pollution (www.unece.org/env/lrtap).

All these national and international measures have brought significant cuts in some forms of air pollution; only the $O_3$ concentration has increased during the last decades (Fig. 1.2).

#### 1.1.3.2 Belgium

Belgium comprises regions with different population densities, i.e., Flanders and Brussels (7 341 521 inhabitants on 13 522 $km^2$) and Wallonia (3

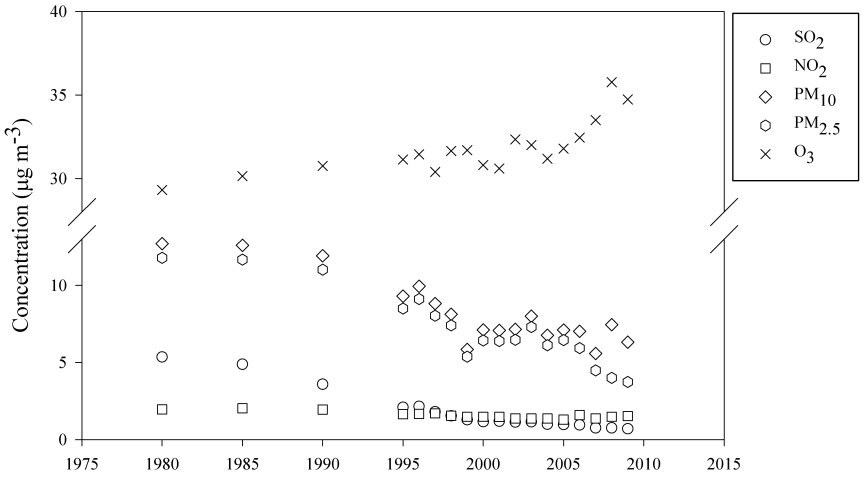


Figure 1.2: The atmospheric concentration of $SO_2$, $NO_2$, $PM_{10}$, $PM_{2.5}$ and $O_3$ in Europe between 1980 and 2009 (www.emep.int)

456 775 inhabitants on 16 844 $km^2$) (Fig. 1.3). Important industrial areas are located at the harbor and docklands of Antwerp (ca. 50 km north of Brussels; mainly petro-chemical industries), Ghent (ca. 60 km northwest of Brussels; steelworks and car assembly) and Liège and Charleroi (ca. 100 km south and ca. 70 km east of Brussels, respectively; steelworks). Flanders, northern Belgium, is crisscrossed by several important highways (E17, E19, E34, E40, E42 and E313) and contains two regions with intensive livestock breeding, which emit high amounts of $NH_3$, i.e., pig farms in the western part and pig and poultry farms in the north-eastern part of Flanders.

The high economic activity and population density lead to the fact that Belgium is one of the most polluted countries of Europe. In 2009, the mean atmospheric concentrations of $NO_2$ (4.3 μg $m^{-3}$), $SO_2$ (1.4 μg $m^{-3}$), $NH_3$ (2.95 μg $m^{-3}$) and $PM_{10}$ (9.3 μg $m^{-3}$) in Belgium were higher than the mean atmospheric European concentrations ($NO_2$ 1.5 μg $m^{-3}$, $SO_2$ 0.7 μg $m^{-3}$, $NH_3$ 1.41 μg $m^{-3}$ and $PM_{10}$ 6.3 μg $m^{-3}$; www.emep.int), while the mean $O_3$ concentration was lower in Belgium (31 ppb) than in Europe (35 ppb).

Policy measures have been taken to improve the quality of fuels and to

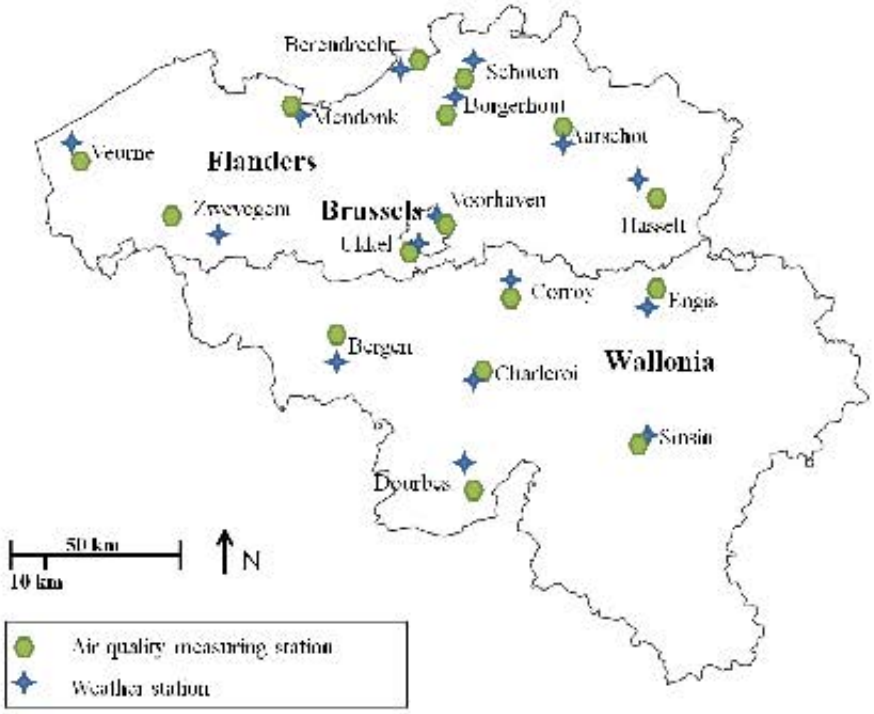


Figure 1.3: Map of Belgium with the location of Flanders, Brussels and Wallonia, together with the air quality measuring stations and weather stations used in the present used in the present thesis

reduce the level of lead in gasoline. Policies promoting cleaner transportation measures and technology, emission limits on vehicular exhaust to meet the national air quality goals and programs designed to increase public awareness about impacts of air pollution are implemented. Consequently, a reduction of $NO_x$ emissions was realized by measures in both industry, which resulted in 75% emission reduction in power plants and 50% in refineries, and traffic, through a combination of avoiding avoidable trips, collective transport, efficient management of the traffic system and greening of vehicle parks (www.lne.be). In addition, Belgium is also member of the Montreal Protocol, and, thus, complies with the European Regulation on substances that deplete the $O_3$ layer, meaning that the production of chlorofluorocarbons needs to be stopped or controlled, as well as the consumption and production of hydrochlorofluorocarbons, hydrobromofluorocarbons, carbon tetrachloride and methylchloroform (www.un.org).

The effort in reducing the emission of air pollutants has led to a substantial decrease of atmospheric $PM_{10}$, $SO_2$ and $NO_2$ concentrations, as shown in Fig. 1.4; the atmospheric $O_3$ concentration increased (Fig. 1.4).


Figure 1.4: The atmospheric concentration of $PM_{10}$, $SO_2$, $O_3$, $NO_2$ in Belgium between 1980 and 2009 (www.emep.int)

Notwithstanding the fact that several policy measures are implemented, a significant percentage of the Belgian population is still exposed to atmospheric pollutant concentrations above the European limit values (Table 1.1). For example, in 2003, more than 80% of the population was exposed to daily mean $PM_{10}$ concentrations higher than 50 μg $m^{-3}$ on more than 35 days. Even more, since 2005, the limit value of $PM_{10}$ has not been respected in eight air quality zones in the Brussels, Flanders and Walloon regions, leading to the decision of the European Commission to take Belgium to the European Court of Justice. The European Commission refused the deferral request of Belgium due to the promotion of diesel cars, the failure to introduce low emission zones, temporary speed limits on smog days, the absence of policies to handle the traffic growth and a misguided spatial policy (www.bondbeterleefmilieu.be).

## 1.2 Impact of air quality on human health and ecosystems

*The quality of daily life depends on many modern conveniences. People enjoy the freedom to drive cars and travel in airplanes for business and pleasure and expect their homes to have electricity and their water to be heated for bathing. People use a variety of products such as clothing, pharmaceuticals and furniture made of synthetic materials. At times, they rely on services that use chemical solvents, such as the local dry cleaner and print shop. Yet, the availability of these everyday conveniences comes at a price, because they all contribute to air pollution (www.epa.gov).*

### 1.2.1 Human health

Exposure to air pollution is associated with several deleterious effects on human health, occurring already for several decades. In 1930 in the Meuse River Valley (Belgium), 63 people died and thousand people were sick due to the high atmospheric $SO_2$ concentration during a temperature inversion (ec.europa.be). Nowadays, there is more emphasis on this negative impact of air pollution on human health (Table 1.2) (Olmo et al. 2011, Kan et al. 2012, Vidotto et al. 2012). It must be noted that these health effects vary from person to person. Elderly, infants, pregnant women, and sick people are more sensitive to the negative effects of air pollution. A chronic exposure to the current $PM_{2.5}$ concentrations in Belgium is also estimated to shorten the healthy life expectancy by almost one year (www.eea.europa.eu). Pope and Dockery (2006) found a death rate of 1.2 deaths per day due to PM exposure in the United States and in Belgium, 5.5% of the mortality was found attributable to $PM_{10}$ concentrations higher than the limit value (Remy et al. 2011).

### 1.2.2 Ecosystems

Forest decline, crop damage and heathland deterioration are only a few examples of the deleterious effect of the increased emission of anthropogenic air pollutants. Forests are sensitive ecosystems and thus highly susceptible to disturbances caused by air pollution (Larcher 2003). In general, air pollution can cause (i) direct damage to leaves, (ii) dysfunctions of stomatal regulation, photosynthesis, growth and development, which can lead to shedding of leaves and needles, water deficiency and decreased resistance to frost and pests and (iii) leaching of mineral substances, which can lead to deficiency of mineral nutrients, soil acidification, release of toxic metal ions

Table 1.2: Human health effects for exposure to $SO_2$, $NO_2$, $O_3$ and PM (after www.epa.gov)

| Pollutant | Health effect |
|---|---|
| $SO_2$ | eye irritation |
| | wheezing |
| | chest tightness |
| | shortness of breath |
| | lung damage |
| $NO_2$ | high susceptibility to respiratory infections |
| | lung irritation |
| | respiratory symptoms |
| | (e.g., coughing, chest pain, difficult breathing) |
| $O_3$ | eye and throat irritation |
| | coughing |
| | respiratory tract problems |
| | asthma |
| | lung damage |
| PM | eye irritation |
| | asthma |
| | bronchitis |
| | lung damage |
| | cancer |
| | heavy metal poisoning |
| | cardiovascular effects |

and changes in the species composition of soil organisms (Larcher 2003). Spatially restricted forest damage, caused by high atmospheric $SO_2$ concentrations in the vicinity of industrial plants, is well-known (Larcher 2003), as well as the serious dieback of forests due to the acidification and eutrophication by increased nitrogen (N) emissions (mainly $NO_2$ and $NH_3$). In Dutch forests, the vitality of Douglas fir (*Pseudotsuga* sp.) and Black pine (*Pinus nigra*) was characterized by a downwards trend, as shown by loss and chlorosis of the foliage, and for Norway spruce (*Picea abies*) a steep decline in vitality has been recorded since 1991 (Van der Eerden 1998). A dramatic shift in species composition of the undergrowth of Dutch pine forests on poor soil also took place; the original moss and lichen-dominated vegetation changed into a grass-dominated vegetation. Shifts in species composition also occurred in lowland heathlands, calcareous grasslands, coastal dunes and wetlands (Sutton et al. 1993), leading to a decreased

biodiversity (Bobbink 1991, Galloway et al. 2003, Krupa 2003, Huang et al. 2012). Heathland species, such as *Calluna vulgaris* and *Erica tetralix*, may compete with, e.g., purple moor-grass (*Molinia caerulea*) at high rates of N deposition, leading to the transition of heathland to grassland. The species diversity of heathlands is also decreased by the acidifying effect of $NH_x$ deposition on species-rich microhabitats with a high pH and on cryptogamic vegetation (Krupa 2003). The degradation of freshwater, estuarine and coastal marine ecosystems is also indicated as a consequence of increased $NH_3$ emission (Camargo and Alonso 2006).

In addition, there is much evidence of crop damage by air pollution. Reduced crop yields (Wahid et al. 1995, Inclan et al. 1999, Ashmore 2005), a decline in numbers of ears, seeds and yield of wheat (Rajput and Agrawal 2005), accelerated senescence (Ashmore 2005), visible injury, which is worse for species with a market value dependent on their visible appearance and reduced protein, sugar, starch and nutrient content of the end-product (Rajput and Agrawal 2005) are well-known adverse effects. All these effects cause serious economic and social implications in regions with problems in maintaining food supplies (Ashmore 2005).

## 1.3 Biomonitoring and bioindication of air quality

*The interest in biomonitoring and bioindication is rising, because unforeseen compounds and interaction effects of air pollutants cannot be evaluated by the currently used physico-chemical air quality monitoring approach. Consequently, biomonitoring/bioindication has been used for supporting the traditional physico-chemical approach (Wuytack et al. 2011).*

### 1.3.1 Definition

Air quality biomonitoring/bioindication is a research domain with a long history and is defined as the response of living organisms to changes in the air quality of their environment and, thereby, obtain information about this air quality (Nali and Lorenzini 2007). Many researchers have used biomonitoring/bioindication as a powerful cost effective and user-friendly tool for filling the gap between the causes and the effects of air quality. The response of a living organism is indeed determined by the antagonistic and/or synergistic interactions between air pollutants and biotic and abiotic factors (Fig. 1.5).

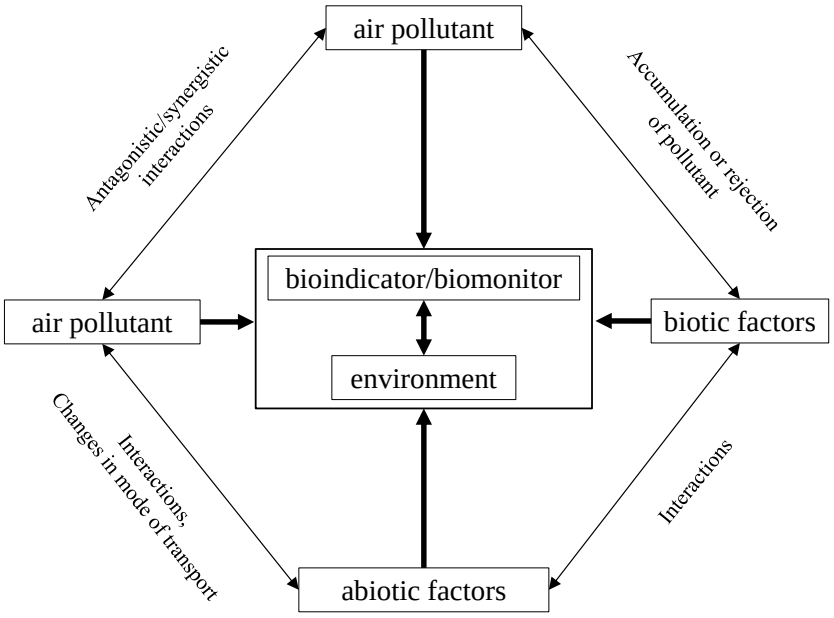


Figure 1.5: Complex ecosystem interactions with regard to air pollutants, and consequences for biomonitoring and bioindication (after Markert 2007)

Biomonitoring is defined as the use of living organisms (biomonitors) to obtain information on quantitative aspects about the environmental quality, while bioindication is the use of living organisms (bioindicators) to gain information about the quality of the environment (Markert 2007). A distinction is also made between, on the one hand, passive and active biomonitors/bioindicators, and, on the other hand, accumulation and impact biomonitors/bioindicators (Markert 2007). In case of a passive biomonitoring/bioindication, the studied organisms are already present in the ecosystem. In case of an active biomonitoring/bioindication, the studied biomonitors/bioindicators are brought into the ecosystem by the researcher for a defined period of time. Accumulation biomonitors/bioindicators accumulate elements from their environment, while impact biomonitors/bioindicators demonstrate effects in response to air pollution exposure (Markert 2007).

Biomonitoring/bioindication is performed on humans (e.g., Schiffmann et al. 2005, Van Dongen et al. 2009, Hohenblum et al. 2012), animals (e.g., Polak et al. 2002, Hendrickx et al. 2003, Hoffmann et al. 2005, Sillanpaa

et al. 2010) and plants (reviewed by Falla et al. 2000). Plants are more frequently used as biomonitor/bioindicator than humans and animals because plants have a broad geographical distribution, are easy to gather and reflect better the local conditions, since they are more sensitive in terms of physiological reaction to the common air pollutants (Falla et al. 2000, Nali and Lorenzini 2007, Raz et al. 2011). The final goal of biomonitoring/bioindication is not to replace the traditional physico-chemical approach, but to supplement it. An integration of the two systems is considered the most appropriate solution for air quality assessment.

### 1.3.2 Biomonitoring and bioindication with plants

#### 1.3.2.1 Lichens and mosses

Lichens were very early designated as organisms to obtain information about air pollution. In 1866, lichens gained their notoriety because of their sensitivity to high $SO_2$ concentrations (Nylander 1866). Lichens represent, indeed, a special vegetal group of fungi and algae or cyanobacteria associations with exceptional resistance and reviviscence capabilities (Falla et al. 2000). Lichens do not have roots, a waxy waterproof cuticle and stomata, which results in the preference to absorb mineral supplies from aerial sources, rather than from the substrate (Reiners and Olson 1984, Wolterbeek 2002).
The use of lichens is twofold:

**Accumulation biomonitoring/bioindication** The porous and absorbent structure of lichens causes a fast penetration of submicronic particles within the thallus, which makes them good bioaccumulatory species (Falla et al. 2000, Loppi and Nascimbene 2010). Moreover, once the particles are absorbed by the thallus, no excretion or removal by leaf litter is possible. Mainly trace elements, such as lead, iron, zinc, radium, fluorine and chlorine are retained. Aboal et al. (2004) showed that *Scleropodium purum* was suitable for biomonitoring several trace elements and *Ramalina celastri* seemed to be suitable to monitor the zinc concentration, associated with motor vehicle traffic and industrial and agricultural activity (Pignata et al. 2007, Bermudez et al. 2009). An extensive overview of the bioaccumulation capacity of several lichens is given in the review article of Conti and Cecchetti (2001).

**Impact biomonitoring/bioindication** The observed increase in abundance of (strictly) nitrophilic lichens due to a rise in bark pH (Frati et al. 2008),

caused by an increased $NH_3$ concentration, revived the interest in the use of lichens as impact biomonitors/bioindicators. Sparrius (2007) recognized indeed that nitrophilous epiphytes are positively correlated with $NH_3$ concentrations, and also Van Dobben and Ter Braak (1998) found a negative correlation between the abundance of nitrophytic lichens and $SO_2$ concentration. However, some disadvantages are related to the use of lichens as impact biomonitors/bioindicators approach: (i) dust and dry conditions also lead to an increase in bark pH, complicating the detection of the effects of nitrogen compounds (Frati et al. 2008) and (ii) using the presence and abundance of lichens to obtain information about air quality requires a specific training (Paoli and Loppi 2008). Changes can also be measured only after damage at community level or at least at species level has occurred (Paoli and Loppi 2008). Therefore, attempts have been made to measure ecophysiological changes (e.g., chlorophyll degradation, chlorophyll fluorescence) and ethylene concentration (Garty et al. 2002, Paoli et al. 2010, Piccotto et al. 2011) for detecting early stress symptoms. Paoli and Loppi (2008) showed that cell membrane damage of *Evernia prunastri*, expressed by changes in electrical conductivity, was a reliable early indicator of deleterious effects caused by geothermal air pollution.

Besides lichens, also mosses are also frequently used species in accumulation biomonitoring/bio-indication studies of, mainly, metallic pollutants and chlorinated hydrocarbons, such as polychloorbifenyl (PCBs) (Wolterbeek 2002). The mosses' capability to be used as bioaccumulators primarily depends on their aptitude to absorb and to fix metallic pollutants as well as their independence concerning ground mineral contributions. However, investigating moss contamination by metals and hydrocarbons requires delicate extraction methods (Wolterbeek 2002). The use of mosses as impact biomonitors/bioindicators is rare, since it is not clear whether chlorosis and/or growth reductions are specific for one or another air pollutant (Garrec and Van Haluwyn 2002). Mosses can also be used as active biomonitors/bioindicators, placed in netted nylon bags, in order to avoid asphyxiation, and are mounted a few meters above the ground (Sun et al. 2009, Ares et al. 2012). The use of moss bags is mainly useful in urban areas where native mosses are scarce or absent and appear to adapt to the surrounding environment (Tyler 1990). Sun et al. (2009) successfully used moss bags to monitor copper, zinc, nickel, lead and mercury, while other studies failed to monitor heavy metals due to the drying out of mosses, leading to a resting phase with no uptake of nutrients (De Temmerman et al. 2004).

#### 1.3.2.2 Higher plants

Higher plants (herbs, shrubs and trees) are often used for the following-up of changes in air quality and the extent of the impact of air pollution (Falla et al. 2000). Various physiological and biochemical processes as well as the morphology and anatomy of shoot and root systems of higher plants can be affected by air pollution, and are, therefore, useful for biomonitoring/bioindication purposes. The tobacco cultivar Bel-W3 proved to be very suitable for biomonitoring the ambient $O_3$ concentration (Falla et al. 2000, Kafiatullah et al. 2012); *Tradescantia pallida* is susceptible to the effects of traffic pollution (Crispim et al. 2012); coniferous trees have been used since 1980 to highlight the pollution impact of $SO_2$ and $O_3$, based on growth variation and chlorosis (Manninen and Huttunen 1995) and the leaf area of higher plants exposed to air pollutants can be reduced by the inhibition of leaf formation, reduced leaf expansion and accelerated leaf abscission (Kozlowski et al. 1991). However, higher plants are unlikely to be the best accumulative biomonitor/bioindicator for air pollutants, when compared to mosses and lichens, due to the presence of a cuticle and stomata in the tissues of higher plants, which makes them less permeable than mosses to air pollutants (Aboal et al. 2004).

Table 1.3 gives an overview of several higher plant species used as bioaccumulator and/or impact biomonitor/bioindicator, along with the response of several leaf characteristics to a single air pollutant or a mixture of air pollutants. The table shows the pollutant-dependent response of the leaf characteristics (Cape et al. 1995), as well as the species-dependent response to air pollution stress.

Table 1.3: Impact biomonitors/bioindicators and accumulation biomonitors/bioindicators with their response to several air pollutants ($O_3$, $NH_3$, sulfur (S), VOC, $SO_2$, $NO_2$, PM, hydrogen fluoride (HF), fluoride (F), black carbon (BC) and lead (Pb)), '+' indicates an increase, '-' indicates a decrease and 0 indicates no change of the plant characteristic

| Species | Air pollutant | Parameter | | Reference |
|---|---|---|---|---|
| **Impact biomonitoring/bioindication** | | | | |
| *Ailanthus altissima* | $O_3$ | callose content | + | Gravano et al. (2003) |
| *Betula pendula* | $SO_2$ | fluctuating asymmetry | + | Kozlov et al. (1996) |
| *Caesalpinia echinata* | $O_3$ | chlorophyll fluorescence | - | Moraes et al. (2004) |
| *Carissa carandas* | HF and $SO_2$ | drop contact angle | - | Pandey (2005) |
| | HF, $SO_2$ | chlorophyll content | - | Pandey (2005) |
| | HF, $SO_2$ | ascorbic acid | - | Pandey (2005) |
| *Crataegus monogyna* | N pollution | $\delta^{15}N$ | + | Marsh et al. (2004) |
| *Daucus carota* | $SO_2$, $NO_2$, $O_3$ | net photosynthesis | - | Tiwari et al. (2006) |
| | $SO_2$, $NO_2$, $O_3$ | stomatal conductance | - | Tiwari et al. (2006) |
| | $SO_2$, $NO_2$, $O_3$ | phenol content | + | Tiwari et al. (2006) |
| | $SO_2$, $NO_2$, $O_3$ | peroxidase activity | + | Tiwari et al. (2006) |
| *Fagus sylvatica* | $O_3$ | chlorophyll fluorescence | - | Bortier et al. (2000) |
| | $O_3$ | net photosynthesis | - | Paoletti et al. (2007) |
| | $O_3$ | drop contact angle | 0 | Paoletti et al. (2007) |
| | $O_3$ | stomatal density | + | Paoletti et al. (2007) |
| *Ficus microcarpa* | $SO_2$, $NO_2$, $O_3$ | peroxidase activity | + | Li (2003) |
| | $SO_2$, $NO_2$, $O_3$ | superoxide dismutase activity | 0 | Li (2003) |

Continued on next page

Table 1.3 – Continued

| Species | Air pollutant | Parameter | | Reference |
|---|---|---|---|---|
| *Fraxinus americana* | $SO_2$, $NO_x$ | leaf area | - | Dineva (2004) |
| *Guaiacum officinale* | $NO_2$ | leaf area | - | Jahan and Iqbal (1992) |
| *Ilex rotunda* | S, F | leaf mass per area | + | Wen et al. (2004) |
| *Machilus chinensis* | S, F | chlorophyll fluorescence | - | Wen et al. (2004) |
| *Magnifera indica* | $SO_2$, $NO_2$, PM | superoxide dismutase activity | + | Tripathi and Gautam (2007) |
| | $SO_2$, $NO_2$, PM | protein content | - | Tripathi and Gautam (2007) |
| | $SO_2$, $NO_2$, PM | sugar content | - | Tripathi and Gautam (2007) |
| *Molinia caerulea* | VOC | drop contact angle | 0 | Cape et al. (2003) |
| *Nicotiana tabaccum* | $O_3$ | necrosis | + | Nali and Lorenzini (2007) |
| *Phaseolus vulgaris* | $NH_3$ | glutamine synthetase activity | + | Pearson and Soares (1998) |
| | $O_3$ | net photosynthesis | - | Schenone et al. (1994) |
| | $O_3$ | stomatal conductance | - | Schenone et al. (1994) |
| *Picea abies* | $SO_2$ | drop contact angle | - | Cape et al. (1995) |
| | $O_3$ | drop contact angle | 0 | Cape et al. (1995) |
| | $NO_x$ | drop contact angle | - | Viskari et al. (2000) |
| | $NO_x$ | stomatal conductance | + | Viskari et al. (2000) |
| | $NO_x$, BC | epicuticular wax amount | - | Viskari et al. (2000) |
| | $SO_2$, $NO_2$, $O_3$ | superoxide dismutase activity | 0 | Nast et al. (1993) |
| *Picea rubens* | $O_3$ | chlorosis | + | Fincher and Alscher (1992) |
| | $O_3$ | glutathione | + | Hausladen et al. (1990) |
| | $O_3$ | tocopherol | + | Hausladen et al. (1990) |

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Table 1.3 – Continued

| Species | Air pollutant | Parameter | | Reference |
|---|---|---|---|---|
| *Pinus densiflora* | $NO_x$ | ethylene emission | + | Kume et al. (2001) |
| *Pinus monophylla* | $SO_2$, $NO_x$ | ring-width growth | + | Thompson (1981) |
| *Pinus ponderosa* | $O_3$ | net photosynthesis | 0 | Beyers et al. (1992) |
| *Pinus sylvestris* | S, heavy metals | fluctuating asymmetry | + | Kozlov and Niemala (1999) |
| | $NH_3$ | chlorophyll fluorescence | + | Bäck et al. (1997) |
| | $NO_2$ | fluctuating asymmetry | 0 | Kozlov et al. (2002) |
| | $SO_2$ | drop contact angle | - | Cape et al. (1983) |
| *Platanus acerifolia* | $SO_2$, $NO_x$ | thickness spongy mesophyll | + | Dineva (2004) |
| *Plantago lanceolata* | urban versus rural | stomatal density | + | Kardel et al. (2010) |
| | urban versus rural | stomatal pore surface | - | Kardel et al. (2010) |
| | urban versus rural | stomatal conductance | - | Kardel et al. (2010) |
| *Pongamia pinnata* | $SO_2$, $NO_2$ | total carbohydrate | - | Bamniya et al. (2012) |
| | $SO_2$, $NO_2$ | total protein | - | Bamniya et al. (2012) |
| | $SO_2$, $NO_2$ | chlorophyll content | - | Bamniya et al. (2012) |
| *Populus nigra* | $O_3$ | chlorosis | + | Novak et al. (2007) |
| | $O_3$ | drop contact angle | - | Schreuder et al. (2001) |
| *Populus x euramericana* | $O_3$ | tannins content | + | Giacomo et al. (2010) |
| | $O_3$ | amount chloroplasts | - | Giacomo et al. (2010) |
| *Robinia pseudoacacia* | $NO_2$, $O_3$ | mesophyll thickness | + | Rashidi et al. (2012) |
| | $NO_2$, $O_3$ | stomatal density | + | Rashidi et al. (2012) |
| *Salix alba* | $NO_2$, $O_3$ | mesophyll thickness | 0 | Gostin and Ivanescu (2007) |

Continued on next page

Table 1.3 – Continued

| Species | Air pollutant | Parameter | | Reference |
|---|---|---|---|---|
| *Salix borealis* | $SO_2$, heavy metals | fluctuating asymmetry | 0 | Zvereva et al. (1997) |
| *Taraxacum officinale* | VOC | chlorophyll a | + | Cape et al. (2003) |
| | $NO_2$ | stomatal pore surface | - | Balasooriya et al. (2008) |
| | $NO_2$ | stomatal density | + | Balasooriya et al. (2008) |
| | $NO_2$ | $\delta^{13}C$ | - | Balasooriya et al. (2008) |
| *Trifolium pratense* | VOC | drop contact angle | 0 | Cape et al. (2003) |
| | $SO_2$, $NO_2$, heavy metals | stomatal density | + | Gostin (2009) |
| | $SO_2$, $NO_2$, heavy metals | stomatal pore surface | - | Gostin (2009) |
| | $SO_2$, $NO_2$, heavy metals | phenol content | + | Gostin (2009) |
| *Triticum* spp. | $O_3$ | yield | + | Rajput and Agrwal (2005) |
| | $O_3$ | growth | - | Wahid et al. (1995) |
| **Accumulation biomonitoring/bioindication** | | | | |
| *Fraxinus excelsior* | Pb, S | | | Aboal et al. (2004) |
| *Lolium perenne* | $NH_3$ | | | Giertych et al. (1997) |
| *Pinus sylvestris* | metals | | | Monaci et al. (2000) |
| *Quercus robur* | trace elements | | | Loppi et al. (1997) |
| *Quercus ilex* | metals | | | Leith et al. (2009) |
| *Quercus pubescens* | trace elements | | | Franzaring et al. (2010) |
| *Salix alba* | heavy metals | | | Vasheggyi et al. (2005) |

#### 1.3.2.3 Biotic and abiotic factors influencing the response of plants to air quality

Analyzing the influence of air quality on the plant performance in the field is complicated by the occurrence of a plethora of (biotic and abiotic) successive and/or simultaneous stresses (Niinemets 2010). The effects of air quality will differ based on the affected organism, the environment and the polluter (Kozlov and Zvereva 2011). In addition, the influence of successive and/or simultaneous stresses is often not additive, but different stresses can interact (Niinemets 2010, Fig 1.5). The interactions can be either negative (antagonistic), implying amplification of the plant response to the given stressor by an additional stress, or positive (synergistic), leading to reduced plant responsiveness to the given stress factor because of an additional stress (Niinemets 2010).

**Affected organism** The tolerance, resistance and/or sensitivity of a species determines whether or not a species can be used in biomonitoring studies and forms the basis of the species-dependent responses of plant characteristics to air quality (Bassin et al. 2009). *Lolium lucidum f. tricolor* is, for example, more tolerant to traffic-related pollution than *Lolium lucidum* (Carreras et al. 1996), and sugar maple (*Acer saccharum*) is relatively tolerant to $O_3$ (Talhelm et al. 2012). *Xanthoria parietina* is a pollution-resistant lichen, while lichen *Flavoparmelta caperata* is considered sensible to gaseous contaminants (Piccotto et al. 2011). Tolerance can be described as the desired resist of an organism to unfavorable environmental conditions, leading to adaptive changes. Resistance is a genetically derived ability to withstand stress, and the sensitivity of an organism is its susceptibility to environmental changes (Markert 2007), which is listed by, e.g., Krupa (2003) for different plant species in terms of $NH_3$ exposure. High sensitivity to air pollution is related to thinner palisade mesophyll layers and a high ratio of spongy to palisade mesophyll cells (Ferdinand et al. 2000). Several other structural and physiological traits, such as leaf area, stomatal apparatus (stomatal number, distribution, size and width), cuticle and mesophyll cell surface, also determine the species' sensitivity, and more specific, the leaf sensitivity (Taylor 1978, Niinemets 2010). Before air pollutants can enter a leaf, they need to overcome an aerodynamic and quasi-laminar boundary layer, i.e., a turbulent and a stable transport zone adjacent to the leaf. The thickness of these boundary layers is determined by the interaction of laminar wind flow and leaf orientation (Larcher 2003, Barber et al. 2004). The presence of hairs on a leaf surface will also affect the thickness of the quasi-laminar boundary layer. Sparse hairs may

increase the surface roughness and turbulence, while a dense mat of hairs will increase the quasi-laminar boundary layer with the depth of the hair mat (Barber et al. 2004 and references herein). Absorption of the air pollutants by the mesophyll cells is only possible when firstly the stomatal and/or cuticular boundary layer, and secondly the mesophyll boundary layer has been overcome (Barber et al. 2004).

The way plants sense stress also varies throughout ontogeny (Milligan et al. 2008). In particular, there is evidence of overall greater resistance to drought, $O_3$ and biotic stress in adult non-senescent trees compared with seedlings and saplings (Niinemets 2010 and references herein). Bystrom et al. (1968) reported a discontinuous cuticular cover in young leaves of beet (*Beta vulgaris*), leading to an increased sensitivity of younger leaves to smog, while Koch et al. (2006) found that younger, not fully developed leaves of cabbage (*Brassica oleracea*) had more epicuticular wax production than the older, fully-expanded leaves. Sunlit leaves of old trees often have a lower photosynthetic rate than sunlit leaves of young trees because of reduced stomatal conductance, causing a lower capacity for defense to or repair of air pollution damage (Niinemets 1999). Factors such as vertical profile of soil water availability, rooting depth, spatial variation in light intensity, canopy proportions of sun and shade leaves and competition with other trees can cause differences in stomatal conductance between juvenile and mature trees (Kolb and Matyssek 2001). It is noteworthy that tolerance to air pollution also depend on the considered plant characteristic, as stated by Schreuder et al. (2001), who found a strong negative effect of $O_3$ on leaf biomass of $O_3$-tolerant poplar (*Populus euramericana)*. A considerable variability in response of leaf characteristics between individual plants of the same species grown under the same conditions, and also between individual leaves on a single plant can occur (Cowart and Graham 1999, Poorter et al. 2009).

**Environment** Air temperature (Kaligaric et al. 2008), relative air humidity (Mortensen et al. 2001), shade (Van Hees and Clerkx 2003), water limitation, altitude and herbivory (Zvereva et al. 1997) have an impact on organisms as well as on the response of plants to air pollution. Ogaya and Penuelas (2007) showed that holm oak (*Quercus ilex*) tends to have more leaves with a higher leaf mass per area unit under high temperature, to maximize photosynthetic gain. High-light plants have sun-type chloroplasts, which possess a higher photosynthetic capacity on a leaf area basis, higher values for chlorophyll a to b ratios, a lower level of light-harvesting chlorophyll a/b proteins, a higher amount of epicuticular waxes and more

stomata with a smaller pore size (Pandey and Nagar 2002, Lichtenthaler et al. 2007). Zaharah and Razi (2009) reported morphological and physiological changes, such as proline accumulation, under water stress. Plants growing along an altitudinal gradient exhibit growth differences, and leaves developed at higher altitude are smaller, have more stomata and more non-glandular hairs to protect against lower temperatures, compared to leaves developed at lower altitude (Kofidis and Bosabalidis 2008). Baker (1974) showed that low air humidity and temperature stimulates wax production, which was confirmed by the findings of Koch et al. (2006). Even the initiation of stomatal primordia can be influenced by air humidity (Ticha 1985) and air temperature (Beerling and Chaloner 1993). In addition, drought stress and wind lead to the closure of stomata and thus reduce the uptake of air pollutants (Chen et al. 1994, Lee et al. 1999, Clark et al. 2000). Leaf temperature, light intensity and relative air humidity all influence the uptake of, e.g., $NH_3$ (Husted and Schjoerring 1996), and according to Kozlov and Zvereva (2011), air temperature increases the harmful impacts of pollution on terrestrial ecosystems.

**Polluter** The effect of air pollutants depends on the time of day when the concentrations are highest. Peak concentrations of atmospheric pollutants occurring before noon, when the stomata are usually fully open, are more harmful than peak concentrations at night (Larcher 2003). If plants have only been exposed to air pollutants for a short period of time during the day, the night can be a time for recovery (Larcher 2003). The amount of and the duration of the exposure to air pollutants will also lead to a variation in plant responses (Kozlov and Zvereva 2011). Peak concentrations of air pollutants during a short-term period cause acute destabilization with acute symptoms such as leaf chlorosis and necrosis as a result (De Temmerman et al. 2002). Long-term exposure to low concentrations of air pollutants can result in chronic destabilization. When chronic destabilization occurs, plants are able to maintain normal function by increasing resistance to further stress or increasing rates of damage repair. However, with chronic destabilization, the capability of a plant to overcome further stress will diminish and an irreversible 'exhaustion' phase will be put in motion, ending with the death of the plant (Larcher 2003). In addition, a combination of $O_3$ and $SO_2$ can cause damage to plants at concentrations that are much lower than that of the air pollutants separately, since stomata will close at a lower $O_3$ concentration when both $O_3$ and $SO_2$ are present (Bläck et al. 1982). The presence of $CO_2$ also reduces the harmful effects of $O_3$ (Pregitzer et al. 2006), and a combination of HF and $O_3$ accelerates the leaf senescence at a concentration in which each single pollutant would exert no adverse effect

on the plant (MacLean 1990).

In conclusion, in field monitoring studies it is very difficult to separate the effects of many intercorrelated biotic and abiotic factors, which makes the interpretation of the adaptation of plant characteristics in terms of air pollution open to some controversy. Thus, as many environmental variables as possible ought to be considered when evaluating plant responses to air pollution.

## 1.4 Aims and outline of the thesis

The general aim of this thesis was to gain insight into the impact of ambient air quality on leaf characteristics of trees, in order to assess the potential of using trees in biomonitoring studies. We focused on the impact of ambient $NO_2$, $O_3$, $SO_2$ and $PM_{10}$ concentrations on saplings of white willow (*Salix alba*), northern red oak (*Quercus rubra*) and Scots pine (*Pinus sylvestris*), as well as the impact of $NH_3$ on leaf characteristics of common oak (*Quercus robur*).

More specifically, the aims of this thesis were:

**a** to assess the potential of common oak as a passive biomonitor to obtain information about the ambient $NH_3$ concentration, and to quantify the response of its leaf characteristics (morphological, anatomical, physiological)

**b** to assess the potential of white willow as an active biomonitor to obtain information about ambient air quality ($NO_2$, $O_3$, $SO_2$ and $PM_{10}$), and to quantify the response of its leaf characteristics (morphological, anatomical, physiological, biochemical)

**c** to assess whether the response of leaf characteristics of white willow to the ambient air quality depends on the exposure duration

**d** to assess whether the response of leaf characteristics to the ambient air quality is species-dependent (fast-growing vs. slow-growing tree species and deciduous vs. coniferous species)

These aims are addressed in the next five chapters. In **Chapter 2**, the potential of common oak as a passive biomonitor to assess the difference in $NH_3$ concentration, by measuring specific leaf area, fluctuating asymmetry, relative chlorophyll content and stomatal resistance, is evaluated. In **Chapter 3**, the potential of white willow as an active biomonitor was evaluated.

Biomass variables and stomatal characteristics were compared between an urban and rural land use class to assess the differences in air quality between both land use classes. In **Chapter 4**, solutions for the shortcomings that surfaced in the exploratory study on active biomonitoring (Chapter 3), i.e., water deficiency and herbivory, are discussed. In addition, the biomonitoring potential of several anatomical, morphological and physiological leaf characteristics of white willow is evaluated and also the influence of exposure time to the ambient air pollution on these leaf characteristics is described. In **Chapter 5**, the effects of the ambient air quality on biochemical leaf characteristics of white willow are described. In **Chapter 6**, the effect of ambient air quality on leaf characteristics of white willow, northern red oak and Scots pine is assessed. The trees were exposed to ambient air during six months and morphological, anatomical and physiological leaf characteristics were measured. Finally, a general discussion and conclusion of this thesis, resulting in a critical overview of the use of trees to monitor ambient air quality and suggestions for further research are given in **Chapter 7**. A schematic overview of the thesis' outline is given in Fig. 1.6.

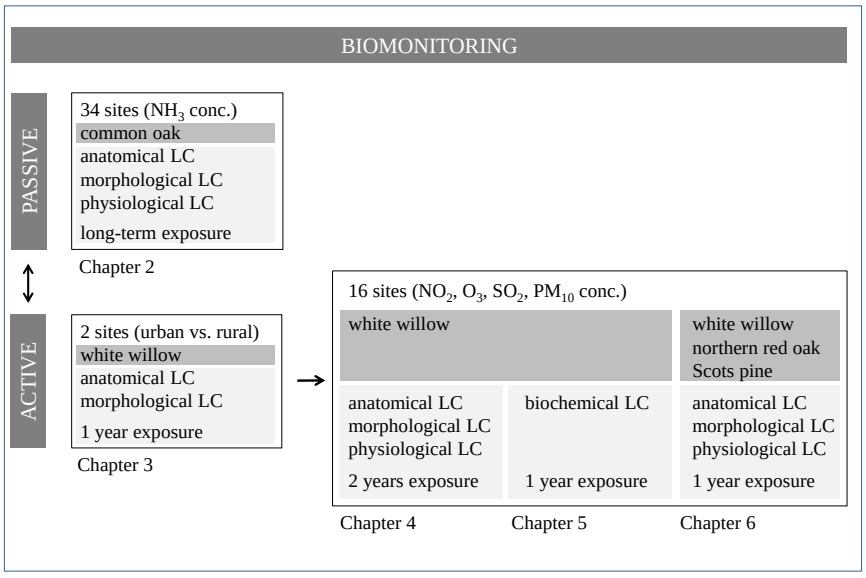


Figure 1.6: Outline of the thesis (LC = leaf characteristics)

# 2

# The use of leaf characteristics of common oak to monitor ambient $NH_3$ concentrations

*After: Wuytack, T., Verheyen, K., Wuyts, K., Adriaenssens, S., Staelens, J., Samson, R. The use of leaf characteristics of common oak (Quercus robur L.) to monitor ambient $NH_3$ concentrations. Submitted to Water, Air and Soil Pollution.*

Biomonitoring the atmospheric $NH_3$ concentrations is generally performed with epiphytic lichens, using species' abundances and/or N concentration as monitoring tools. However, the potential of leaf characteristics of trees to monitor the atmospheric $NH_3$ concentration has remained largely unexplored. Therefore, we performed a passive biomonitoring study with common oak at 34 sampling locations in the near vicinity of livestock farms, located in Flanders (northern Belgium). We aimed at evaluating the potential of specific leaf area, leaf area fluctuating asymmetry, stomatal resistance and chlorophyll content of common oak to monitor a broad range of $NH_3$ concentrations (four monthly average of 1.9 to 29.9 $\mu g\ m^{-3}$). No significant effects of ambient $NH_3$ on the abovementioned leaf characteristics were revealed, which demonstrates the inabil-

ity of using the leaf characteristics of common oak to monitor the ambient $NH_3$ concentration. Probably, differences in climate, soil characteristics, concentrations of other air pollutants and/or genotypes confounded the influence of $NH_3$.

## 2.1 Introduction

During the last decades, anthropogenic activities have led to an increased atmospheric concentration of reactive N (Krupa 2003), which includes $NH_3$, ammonium ($NH_4^+$), $NO_x$, nitrous oxide ($N_2O$), nitrous acid ($HNO_2$), nitric acid ($HNO_3$), and organic N compounds. Reduced N ($NH_x$, i.e., $NH_3$ and $NH_4^+$) originating from intensive stock breeding, is mainly responsible for large scale eutrophication and acidification (Krupa 2003, Pitcairn et al. 2003). Almost 30% of emitted $NH_3$ is converted to $NH_4^+$, which is then either removed by wet or dry deposition (Krupa 2003, Paoli et al. 2010). Unaltered $NH_3$ is deposited in the vicinity of the source, leading to a trend of decreasing atmospheric $NH_3$ concentration away from the source (van Herk et al. 2003, Frati et al. 2007).

Atmospheric $NH_3$ is a major N source, increasing growth in N limited habitats (Krupa 2003). For example, high atmospheric $NH_3$ concentrations acted as a nutrient for *Brassica oleracea* (2.8 mg $NH_3$ $m^{-3}$; Castro et al. 2008) and poplar (0.1 mg $NH_3$ $m^{-3}$; van Hove et al. 1989). However, atmospheric $NH_3$ can also be phytotoxic when the plant's capacity of detoxification is exceeded, causing acute or chronic damage (see §1.3.2.3). Acute damage is reflected in bleached grey foliage, reduced growth and even necrosis of leaf tissue (van der Eerden 1982, Sheppard et al. 2008). On a longer time scale, high atmospheric $NH_3$ concentrations and consequently high $NH_x$ deposition can cause chronic damage, such as ecosystem N saturation with enhanced N leaching to ground water (Gundersen et al. 2006), increased NO emission (Fenn et al. 1996) and a shift in species composition from N sensitive species (e.g., mosses) to nitrophilic species (e.g., some graminoids) (van der Eerden et al. 1998, Pitcairn et al. 2003, see §1.2.2). In order to minimize these negative effects of atmospheric $NH_3$ on ecosystems, $NH_3$ emission abatement policies were developed and pollution control techniques were applied (see §1.1.3.1). To quantify the contribution of these control techniques in reducing the $NH_3$ emission, determination of atmospheric $NH_3$ concentration is needed. Unfortunately, $NH_3$ is not routinely measured by air quality monitoring stations or passive samplers, and, therefore, the use of accumulation and/or impact biomonitors/bioindicators (see §1.3.1) provides a less costly alternative to ob-

tain information about the atmospheric $NH_3$ concentrations, and can also show whether the critical level of 8 µg $NH_3$ $m^{-3}$ is exceeded (Pitcairn et al. 2003, see §1.1.3.1). As a consequence, there has been growing interest in biomonitoring of atmospheric $NH_3$ concentrations by using lichens (see §1.3.2.1). In addition, biomonitoring air pollution with leaf characteristics of plants is becoming more and more applied (Bortier et al. 2001, Kardel et al. 2012, Table 1.3), but these studies mainly deal with the biomonitoring of, e.g., $O_3$, $NO_x$, $SO_2$ or heavy metals. Only a few studies have investigated the relationship between the atmospheric $NH_3$ concentration and tree or leaf characteristics such as visible leaf injury (Van der Eerden et al. 1991), stomatal conductance (van Hove et al. 1989), erosion of the epicuticular wax layer and growth of trees (Dueck et al. 1990). To our knowledge, an assessment of the potential of anatomical, morphological and physiological tree leaf characteristics for biomonitoring the atmospheric $NH_3$ concentrations has not yet been reported. Therefore, the aim of this study was to assess the relationship between the annual mean $NH_3$ concentration and leaf characteristics of common oak, i.e., specif leaf area (SLA), leaf area fluctuating asymmetry (FAA), relative chlorophyll content (RCC) and stomatal density (SD) and pore surface (SPS) of common oak. We hypothesized that SLA, FA and SD would increase with increasing atmospheric $NH_3$ concentration (Velickovic and Perisic 2006) while RCC and SPS would decrease (Joshi and Swami 2009).

## 2.2 Materials and methods

### 2.2.1 Study area and experimental design

In 2008, a biomonitoring network was developed in the vicinity of livestock farms in Flanders to analyze the effectiveness of epiphytic lichens as a bioindicator for atmospheric $NH_3$ concentration. Flanders, northern Belgium (between 51° and 60°N, and 2.60° and 5.8°E), is characterized by important industrial areas, several international highways and two regions with intensive livestock breeding (see §1.1.3.2), leading to a high atmospheric $NH_3$ emission. The abundance of lichens on common oak and hybrid poplar (*Populus x canadensis*) was determined and related with the distance from the livestock farms, which was used as a proxy for the atmospheric $NH_3$ concentration (Van den Broeck et al. 2009). The network covered 144 locations, characterized by a different $NH_3$ load, and were selected at more than 20 km of the North Sea coast to avoid the influence of sea spray. At 100 locations the monthly atmospheric $NH_3$ concentration was measured from January 2008 till January 2009 with diffusive Radiello

samplers (polyethylene cartridge impregnated with phosphoric acid). They were installed at a height of 2.5-3 m on the north-eastern side of the trees to prevent contamination by wet deposition of $NH_4^+$, since the main wind direction in the region is southwest (Van den Broeck et al. 2009).

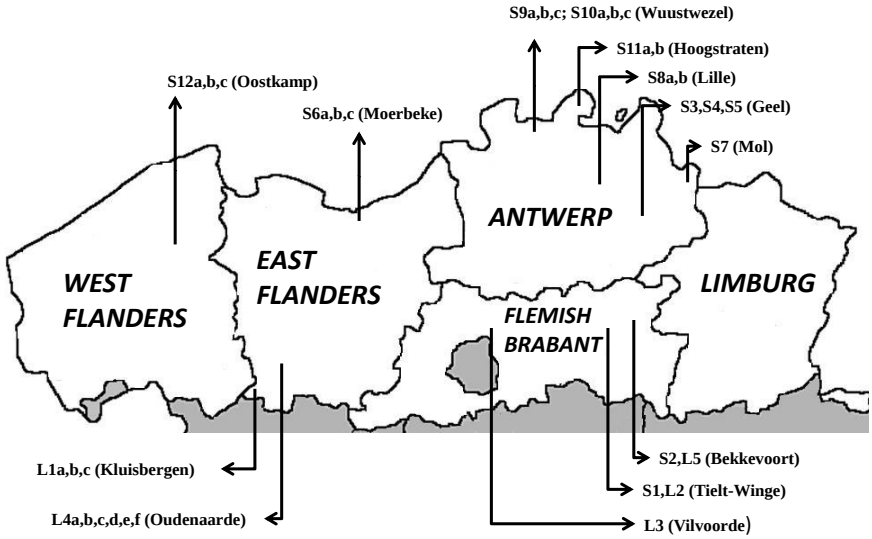


Figure 2.1: Location of the sampling areas in Flanders, indicated by S (sandy soil) and L (sandy loam soil) and followed by a number, with one or more sampling locations in each area, indicated by a letter

From this biomonitoring network, we selected 34 locations (Fig. 2.1) that were spread over Flanders and over the range of atmospheric $NH_3$ concentrations, to perform a passive biomonitoring study with common oak. Only the sandy and sandy loam soil types, as determined from soil maps (geovlaanderen.be), were included in this selection to minimize the possible confounding effect of soil type. Soil was covered with grass vegetation. The atmospheric $NH_3$ concentration measured from April until July 2008 was considered as a representative measure of the atmospheric $NH_3$ concentration during our fieldwork period (April until July 2009). At each location, terminal leaves of six southerly orientated second-order branches of maximum three adjacent trees (< 5 m distance) were sampled. We used the tree to which the Radiello sampler was attached and, if possible, the

trees left and/or right from the Radiello sampler tree. Subsequently, morphological (SLA and FAA), anatomical (SD and SPS) and physiological (RCC) leaf characteristics were determined at the end of July 2009.

## 2.2.2 Data acquisition

### 2.2.2.1 Air quality data

Within one week after exposure, $NH_3$ samplers were desorbed with ultrapure water that was analyzed using spectrophotometry. Air concentrations were calculated from the ion amounts in the desorption water using a temperature-dependent diffusivity based on a laboratory validation (Swaans et al. 2005). Regarding the precision of the samplers, the coefficient of variation between duplicated biweekly measurements at nine sites in 2005 was on average 3.3% (Staelens et al. unpublished data). To describe the atmospheric $NH_3$ pollution at each location, the mean $NH_3$ concentration from April (start of growing season) till July 2009 (harvest) was calculated.

### 2.2.2.2 Morphological leaf characteristics

Per branch we collected five fully developed and undamaged leaves to calculate SLA ($cm^2\ g^{-1}$, n = 30 per sampling location). From each leaf, two leaf discs (0.623 $cm^2$) were punched out at both sides of the midrib and in the middle of the leaf. The leaf discs were dried (48 h at 70°C), weighed (B310S, Sartorius, Germany; ± 0.001 g) and SLA was calculated per leaf according to Eq. 2.1.

$$\text{SLA} = \frac{\text{leaf area}}{\text{leaf biomass}} \tag{2.1}$$

To calculate FAA [1], we randomly collected ten fully developed and undamaged leaves per branch (n = 60 per sampling location). A small sample size was chosen, due to the goal of this research in finding a time- and cost-effective biological monitor. Comparable sample sizes were used by, e.g., Hodar (2002) and Hagen et al. (2008). After harvest, each leaf was sliced along the middle of the mid vein and the surface area of both right (RA, $cm^2$) and left (LA, $cm^2$) lamina sides was measured using a leaf area meter (Li-3100 area meter, Li-COR, Lincoln, Nebraska, accuracy 0.01 $cm^2$). Leaves were immediately measured after harvest or were stored in a cooling box and scanned within 24 hours. RA and LA were used to calculate

[1] Fluctuating asymmetry (FA) is defined as a slight, non-directional, deviation from perfect symmetry of a bilateral character, due to genetic and/or environmental stress (Palmer and Strobeck 1986)

FAA. Since measurement errors can complicate tests involving FA analyses, the accuracy of the measurements needs to be tested (Kozlov et al. 2009) and, therefore, RA and LA were each measured ten times for 25 randomly collected leaves, on different dates and in random order to reduce bias.

#### 2.2.2.3 Anatomical and physiological leaf characteristics

Since common oak has hypostomatous leaves, stomatal characteristics were determined at the left and right abaxial leaf side of six fully developed and undamaged leaves at each location (n = 12 per sampling location). Stomatal imprints were made on attached leaves by applying colourless nail varnish and peeling off the surface with a transparent adhesive tape which was then fixed on a microscope slide. Stomatal imprints were analyzed with a light microscope (Wild Leitz GmbH 020-505.030 CX41RF, Olympus, Germany) connected with a camera at a magnification of 40x10 and imaging software (CellD, Imaging Software, Olympus, Germany). First, the statistical minimal number of microscopic fields ($N_{min}$) that should be counted on a single imprint was calculated, using the Student-t-test (Eq. 2.2).

$$N_{min} = \frac{t^2_{0.0025}(24)}{\frac{x^2}{100}} \times S^2 \tag{2.2}$$

where $t^2_{0.0025}(24)$ is the t-value for (25-1) degrees of freedom, with a threshold value of p = 0.05; x the mean number of stomata in the 25 microscopic fields and S the standard deviation. Subsequently, the number of stomata was counted on $N_{min}$ microscopic fields for each stomatal print. SD (i.e., the number of stomata per $mm^2$ leaf area), SPS (i.e., the surface area of a widely opened stomatal pore, in $\mu m^2$) and stomatal resistance ($R_S$, in s $m^{-1}$) were determined (Eq. 2.3).

$$R_S = \frac{4l}{n\pi LWD} + \frac{L+W}{4nLWD} \tag{2.3}$$

with $R_s$ the minimal theoretical stomatal resistance (s $m^{-1}$); L the length (m, Fig. 3.2) and W the width (m, Fig. 3.2) of widely opened stomata; l the depth of the stomatal pore (m); n the stomatal density ($m^{-2}$) and D the diffusion coefficient of water vapor in the air (0.242 $10^{-4}$ $m^2$ $s^{-1}$ at 20°C). The depth of the stomatal pore was assumed as 10 μm (Olyslaegers et al. 2002).

Finally, on each branch we collected four fully developed and undamaged

leaves to measure RCC on both left and right leaf side (n = 48 per sampling location). The leaves were washed with distilled water to remove small particles and air dried, after which RCC was immediately measured using a CCM-200 plus Chlorophyll Content Meter (Opti-Sciences, ADC Bioscientific). The CCM-200 has the advantage of being rapid, nondestructive and pocket-portable. Moreover, according to Cate and Perkins (2003), RCC values are strongly correlated with chlorophyll concentrations determined by means of a spectrophotometer.

#### 2.2.2.4 Growth parameters

Circumference (m) at breast height (1.3 m) and tree age (year), were measured to take the possible confounding effect of growth differences into account. To determine age, the trees were cored using a Pressler corer at the trunk base to obtain two perpendicular core samples per tree. Circumference ranged from 0.58 m to 2.48 m and tree age ranged from 16 years to 89 years. Since circumference and age were significantly correlated ($p < 0.001$, $R^2 = 0.53$), the number of variables was reduced by using the ratio of circumference to age (growth rate, cm $yr^{-1}$).

### 2.2.3 Statistical analysis

Before comparing FAA between the sampling locations, we first conducted preliminary analyses to find out (i) the degree of measurement error, (ii) whether leaf asymmetry can be defined as FA rather than directional asymmetry (DA) or antisymmetry (AS) and (iii) the importance of size scaling. DA and AS are considered as confounding factors of FA, since they have an unknown genetic component (Palmer and Strobeck 1986).

To test the accuracy of the measurements, the statistical methodology of Hodar (2002) and Roy and Stanton (1999) was used. Within-subject and between-subject variability were compared and a Pearson correlation between the original and remeasured data was performed. DA is characterized by a normally distributed (right (R) - left (L)), where the mean departs significantly from zero. Therefore, presence of DA within each trait was checked by testing whether the mean of signed (R-L) is significantly different from zero, using a one-sample t-test. AS is associated with a platykurtic or bimodal distribution of (R-L) with a mean of zero. Deviations from normality or leptokurtic distributions of signed (R-L) were assessed using kurtosis (Bonett-Seier) and a Kolmogorov-Smirnov test, for detecting AS. Since traits that grow larger have more 'opportunity' to develop larger absolute differences between left and right lamina sides, size-dependency of

asymmetry needs to be analyzed by using a regression of unsigned (R-L) on (R+L)/2 for all leaves according to the method of Raz et al. (2011). When a positive size-dependent asymmetry is present, a multiplicative error model is probably appropriate since plant leaves seems likely to grow according the active tissue growth model (Graham et al. 2003). The traditional way to correct for positive size dependency, by either dividing |R-L| by (R+L)/2 or by simply using |log R - log L|, often generate an over-correction or negative size-dependent asymmetry since measurement error is additive (Raz et al. 2011). If the measurement error cannot be removed beforehand (as is the case in our research), the only resource for compensating the over-correction, is a power (Box-Cox) transformation of the raw data (Raz et al. 2011). The final step in the preliminary analysis is to check the independence of the traits by using a Pearson correlation on the signed (R-L) values, since the advantage of combining traits decreases as the degree of correlation between traits increases (Leung et al. 2000).

Because of the hierarchical nature of the data, we used linear mixed models to relate the leaf characteristics to the set of explanatory variables, i.e., atmospheric $NH_3$ concentration, soil type, growth rate, atmospheric $NH_3$ concentration x soil type and $NH_3$ concentration x growth rate. Several leaves were sampled on the same branch, multiple branches occurred on a tree and multiple trees were analyzed per location; hence, branch level was nested within tree level, which was nested within location level. Following Zuur et al. (2009), we first determined the optimal random model structure by stepwise deleting the lowest hierarchical level (starting with 'branch') and comparing the model with and without the deleted random effect using a likelihood ratio test. A linear model is preferred when only the level 'location' remains and when the Akaike Information Criterion (AIC) value is lower for the linear model compared to the mixed effect model. Next, the fixed effects structure was optimized starting from a model that included all explanatory variables and the first order interactions with the atmospheric $NH_3$ concentration. Model terms with non-significant ($p > 0.05$) parameter estimates and non-significant contributions to the overall model (likelihood ratio test, $\chi^2$) were successively removed, starting with the interaction terms. The null model is taken as the optimal fixed effects model when no explanatory variables contributes significantly to the overall model. The final model was refitted using Restricted Maximum Likelihood (ML) estimates before any conclusion was made, since estimates of variance components based on ML are biased (Pinheiro et al. 2009). All analyses were performed on the 5% level of significance and run with R 2.10.1 (R Development Core Team 2009) using the nlme package (Pinheiro et al. 2009) to

fit the linear mixed models.

## 2.3 Results

### 2.3.1 Air quality

In 2008, the monthly $NH_3$ concentration was highest during February and May and lowest during November and December. This variation is caused by the fact that fertilization of agricultural fields is prohibited in the study region from 1 September to 15 February so that much fertilizer is applied at the end of February. Also, during May, fields are fertilized before sowing of maize and after mowing of grass. The critical level for $NH_3$ of 8 μg $m^{-3}$ was exceeded in the locations Wuustwezel and Hoogstraten (in the north, Fig. 2.1) and Oostkamp (in the west, Fig. 2.1), with a maximum exceedance of 16.53 μg $m^{-3}$ in Wuustwezel (S10a; Fig. 2.1). The mean $NH_3$ concentration for each sampling location is given in Table 2.1.

### 2.3.2 Leaf characteristics

For SLA, the fixed effect model was optimized by first removing the interaction term 'growth rate x $NH_3$ concentration', since it did not significantly contribute to the model ($p = 0.321$, $R^2 = 0.985$). On the contrary, the interaction term 'soil type x $NH_3$ concentration' improved the model ($p = 0.018$), indicating a counteracting effect of 'soil type'. However, the significant contribution of this interaction term in the fixed effect model was caused by a leverage effect, caused by two locations on loamy sand, L2 and L5 (Fig. 2.1). Therefore, these two locations were removed and the statistical analysis was redone.

For FAA, first of all, the presence of DA, AS and size dependency needed to be investigated. The one-sample t-test revealed no significant difference ($p = 0.336$) between LA and RA, indicating a lack of DA. The Shapiro-Wilk test revealed that the (R-L) distribution of leaf area significantly deviated from normality ($p < 0.001$) and the positive kurtosis ($\gamma = 3.437$) revealed a leptokurtic distribution, indicating a lack of AS. Unsigned (RA-LA) values positively correlated with size trait (RA+LA)/2 ($r = 0.500$, $p < 0.001$, $n = 1928$). Therefore, we log-transformed the raw data of RA and LA and regressed |log RA - log LA| on (log RA + log LA)/2 to examine negative size dependency. Based on the slope of the regression (-0.0804) and the poor fit ($R^2 = 0.0005$), we concluded that the log-transformation of the raw data caused no negative size-dependency and, therefore, FA can be calculated

by |logRA - logLA|. The precision of the measurements was tested by repeated measurements of leaf area of 25 leaves, resulting in a within-subject variability of 0.0059 for LA and 0.0037 for RA and a between-subject variability of 0.257. Moreover, significant relations were present between the measurement series ($R^2$ = 0.814 to 1.000 for LA and $R^2$ = 0.984 to 1.000 for RA; $p < 0.001$), demonstrating high repeatability and reliability of the leaf area measurements. We used a linear model to analyze FAA instead of a mixed model, based on the difference of 40.7 in AIC value. The FAA differed significantly between the sampling locations ($p < 0.001$, t = 17.423) and ranged from 0.00025 to 0.34979. This difference was significantly related to the interaction terms 'growth rate x $NH_3$ concentration' ($p < 0.001$, t = 4.710) and 'soil type x $NH_3$ concentration' (p = 0.004, t = 2.894). But, again, the significant contribution of 'soil type x $NH_3$ concentration' was achieved by a leverage effect, caused by the same two locations L2 and L5 on sandy loam as for SLA. Therefore, the statistical analysis was repeated after omitting these two locations.

Because of the significant positive correlation ($p < 0.001$, n = 204) between SD, SPS and $R_S$ of the left and right lamina side ($R^2$ = 0.59, $R^2$ = 0.54, $R^2$ = 0.46, respectively) and the significant negative correlation ($p < 0.001$; n = 204) between the mean $R_S$ and the mean SD ($R^2$ = 0.56) and the mean SPS ($R^2$ = 0.58), only the mean $R_S$ per leaf was used in the statistical analysis (Table 2.1). Mean SD ranged from 409 to 566 stomata $mm^{-2}$ and mean SPS ranged from 50.3 to 109.4 $\mu m^2$. Similarly, RCC of the left and right lamina side were positively correlated ($p < 0.001$, n = 805) so that the mean RCC per leaf was used in further analysis (Table 2.1).

The results of the mixed or linear model are given in Table 2.2. Based on these results, we can conclude that the atmospheric $NH_3$ concentration could not explain the variability in SLA (Fig. 2.2A), RCC (Fig. 2.2C) and $R_S$ (Fig. 2.2D). Only for FAA, the linear model showed a significant effect of the atmospheric $NH_3$ concentration, growth rate and the interaction term 'growth rate x $NH_3$ concentration', but these results could not be confirmed when plotting the $NH_3$ concentration against FAA (Fig. 2.2B).

Table 2.1: Mean ammonia concentration ($NH_3$, µg $m^{-3}$) (April until July 2008), specific leaf area (SLA, $cm^2$ $g^{-1}$), leaf area fluctuating asymmetry (FAA, -), relative chlorophyll content (RCC, -) and stomatal resistance ($R_S$, s $m^{-1}$) for each sampling area, with one or more sampling locations in each area

| Soil type | Location | $NH_3$ | SLA | FAA | RCC | $R_S$ |
|---|---|---|---|---|---|---|
| Sandy | S1 | 3.17 | 119.8 | 0.074 | 12.98 | 19.68 |
| | S2 | 4.24 | 99.1 | 0.048 | 17.96 | 18.2 |
| | S3 | 4.3 | 115.3 | 0.066 | 19.19 | 20.14 |
| | S4 | 6.63 | 110 | 0.058 | 16.1 | 20.17 |
| | S5 | 6.76 | 120.7 | 0.067 | 15.21 | 22.72 |
| | S6a | 6.85 | 155.7 | 0.062 | 17.35 | 24.29 |
| | S6b | 7.35 | 106.3 | 0.066 | 17.45 | 25.21 |
| | S6c | 8.56 | 125.4 | 0.069 | 15.14 | 25.15 |
| | S7 | 6.91 | 96.4 | 0.061 | 24.36 | 20.57 |
| | S8a | 9.28 | 119.5 | 0.061 | 23.69 | 19.5 |
| | S8b | 10.74 | 133.4 | 0.059 | 14.79 | 18.73 |
| | S9a | 15.22 | 123.9 | 0.062 | 15.72 | 18.45 |
| | S9b | 13.08 | 91 | 0.064 | 17.52 | 21.35 |
| | S9c | 15.66 | 101.6 | 0.064 | 17.28 | 20.48 |
| | S10a | 25.93 | 103.8 | 0.065 | 17.79 | 20.01 |
| | S10b | 17.02 | 92.6 | 0.075 | 25.63 | 20.65 |
| | S10c | 19 | 99 | 0.077 | 17.53 | 21.39 |
| | S11a | 24.69 | 121.5 | 0.086 | 17.19 | 20.66 |
| | S11b | 17.36 | 106.2 | 0.074 | 22.75 | 19.96 |
| | S12a | 19.21 | 141.6 | 0.054 | 18.78 | 21.81 |
| | S12b | 18.16 | 96.1 | 0.064 | 21.25 | 19.05 |
| | S12c | 29.92 | 95.6 | 0.067 | 16.75 | 18.76 |
| Sandy loam | L1a | 6.34 | 101.4 | 0.061 | 16.46 | 21.85 |
| | L1b | 6.42 | 90.4 | 0.053 | 17.98 | 21.2 |
| | L1c | 5.69 | 102.5 | 0.084 | 12.77 | 20.04 |
| | L2 | 1.89 | 154.2 | 0.057 | 26.78 | 20.94 |
| | L3 | 5.35 | 97.9 | 0.058 | 21.98 | 20.29 |
| | L4a | 6.01 | 115.9 | 0.072 | 17.8 | 22.00 |
| | L4b | 5.51 | 116.5 | 0.072 | 14.45 | 20.98 |
| | L4c | 5.51 | 105.4 | 0.055 | 19.69 | 22.81 |
| | L4d | 5.27 | 103 | 0.064 | 11.47 | 20.61 |
| | L4e | 5.75 | 107.4 | 0.061 | 13.17 | 20.72 |
| | L4f | 5.57 | 111.4 | 0.057 | 20.31 | 19.61 |
| | L5 | 7.45 | 92.9 | 0.057 | 29.52 | 19.72 |

Table 2.2: The contribution of each level (branch and tree) in the optimal random model and the contribution of the explanatory variables (single and interaction) in the optimal fixed effect model indicated by the p and $\chi^2$ or F value for specific leaf area (SLA, $cm^2$ $g^{-1}$), leaf area fluctuating asymmetry (FAA, -), relative chlorophyll content (RCC, -) and stomatal resistance ($R_S$, s $m^{-1}$); n/a: not applicable

| | Variation at each level of the random model | | | Explanatory variable | Explained variability |
|---|---|---|---|---|---|
| | Location | Tree | Branch | | |
| SLA | 56 | 12 | 32 | none (p = 0.145, $\chi^2$ = 5.399) | n/a |
| FAA | 0.1 | 90 | n/a | growth rate x $NH_3$ (p ¡ 0.001, F = 2.00)<br>growth rate (p < 0.001, F = 11.82)<br>$NH_3$ (p < 0.001, F = 9.73) | 60 |
| $R_S$ | < 0.1 | 52 | n/a | none (p = 0.666, $\chi^2$ = 1.47) | n/a |
| RCC | 37 | 35 | 10 | none (p = 0.892, $\chi^2$ = 0.618) | n/a |

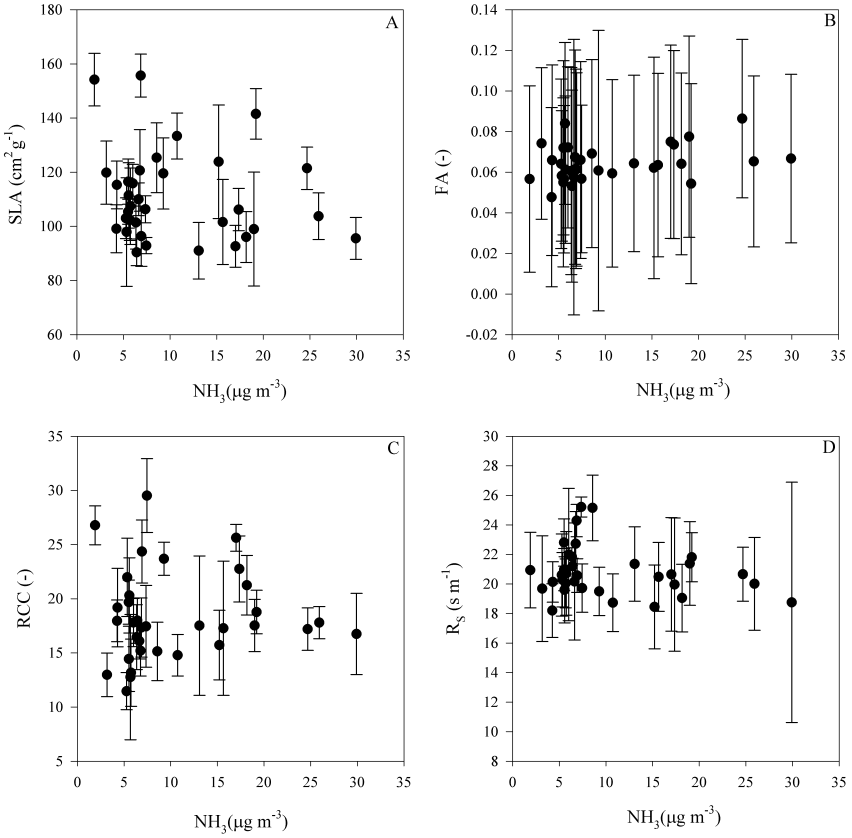


Figure 2.2: Relationship between the mean ammonia ($NH_3$) concentration and mean (a) specific leaf area (SLA), (b) leaf area fluctuating asymmetry (FAA), (c) relative chlorophyll content (RCC) and (d) stomatal resistance ($R_S$)

## 2.4 Discussion

### 2.4.1 Within and between-plant variability

The total observed variability of SLA, RCC and $R_S$ of common oak was explained by a relative high within-plant (24%, 10% and 44%, respectively) and between-plant variability (12%, 35% and 52%, respectively). In contrast, for FAA only a small part of the variation was explained by the within-plant (1.4%) and between-plant (4.1%) variability. The observed within-

plant variability of SLA was low compared with the results of Poorter et al. (2009), where a twofold difference of SLA within a single plant was generally present. Within-plant variability of leaf characteristics can occur due to a gradient in light, air temperature, air humidity and wind velocity, present in larger trees (Poorter et al. 2009 and references herein) and plant specific factors, such as age, development stage and the position of the leaf on the plant (Gunn et al. 1999). Leaves adapt to lower irradiance by producing thinner and larger leaves, resulting in a higher SLA, and by an increased $R_S$ as a consequence of optimizing $CO_2$ uptake and reducing water loss by transpiration (Barber et al. 2004, Gratani et al. 2006). Moreover, sun leaves possess a higher chlorophyll content compared to shade leaves due to the high irradiance adaptation response of the photosynthetic response apparatus (Sarijeva et al. 2007), while under too much light leaves become chlorotic (Larcher 2003). High air temperature leads to a decrease in chlorophyll content, in order to increase reflectance and decrease the intercepted light, but also as a protection mechanism against photodestruction (Gratani et al. 2011). An increase in air temperature also causes a decrease in $R_S$ up to the optimal air temperature, which can be seen as an adaptation to reduce evapotranspirational water loss (Beerling and Chaloner 1993, Barber et al. 2004). Furthermore, genetic differences between the sampled trees can lead to the observed between-plant variability as well. This genetic variability may underlie small differences in SLA (Bonser et al. 2010) and stomatal responses (Pä¨akkönen et al. 1993), but can also lead to larger differences of FAA between clones than between treatments (Dimitriou et al. 2006).

### 2.4.2 Between-site variability

In case of FAA, RCC and $R_S$, the between-site variability explained a small part of the total variability ($< 0.1\%$), while the between-site variability of SLA explained 56% of the total variability. The between-site variability of FAA was related to the interaction between growth rate and mean $NH_3$ concentration. However, this interaction is difficult to interpret, due to the continuous character of the two explanatory variables. Probably, as stated by Martel et al. (1999), the faster growing oaks developed a higher developmental instability due to an increased energy demand to produce larger leaves, which can give rise to an increased FAA. However, regardless the significant between-site variability, the low measurement error and the high statistical power (91%), the very high residual variability (95%) raises the question whether FAA needs to be interpreted as noise rather than as a true signal.

For all other leaf characteristics, the between-site variability was not related to the mean $NH_3$ concentration (Fig. 2.2A-D), even though the critical level of 8 μg $NH_3$ $m^{-3}$ was exceeded at several sampling locations. The incidence of such an exceedance may indicate that direct adverse effects on plants may occur, as stated by Posthumus (1988). However, no adverse effect of $NH_3$ on the measured leaf characteristics of mature common oak could be detected in this study. The lack of an adverse effect confirms the statement of Cape et al. (2009) that 'exceedances of the critical level do not guarantee that an (adverse) effect will be observed, due to the presence of other environmental stressors and their interaction with $NH_3$ concentration'. Indeed, plants are exposed to a broad range of uncontrolled and/or unmeasured variables, which interact in an unknown synergistic or antagonistic way, making it difficult to separate the effects of intercorrelated variables. Therefore, biomonitoring studies need to take into account the possible single and interacting effects of other atmospheric pollutants, such as $SO_2$, $NO_2$ and $O_3$ on SLA (e.g., Bassin et al. 2009), chlorophyll content (e.g., van Hove et al. 1992), FAA (e.g., Chapter 4) and stomatal characteristics (e.g., Elagoz et al. 2006). Atmospheric pollutants can disturb stomatal control mechanisms (Robinson et al. 1998), since plants optimize their stomatal closure efficiency by increasing stomatal density and decreasing stomatal pore surface as a response to air pollution (Elagoz et al. 2006, Kardel et al. 2010, Chapter 3). Chlorophyll degradation can occur as a response to $O_3$ (Calatayud et al. 2011), power plant pollution (Sharma and Tripathi 2009) and PM (Kuki et al. 2008). van Hove et al. (1991) also demonstrated that moderate $NH_3$ concentrations can alleviate the inhibitory effect of $SO_2$ on photosynthesis, indicating a synergistic interaction between $NH_3$ and $SO_2$. In contrast, $O_3$ can increase the plant's sensitivity to $NH_3$ by decreasing the amount of energy available for $NH_3$ assimilation, indicating an antagonistic interaction between $NH_3$ and $O_3$ (Krupa 2003). However, the concentration of other air pollutants was not measured in our study at each sampling location, making it impossible to evaluate the share of these pollutants and their interactions in the significant between-site variability of the leaf characteristics of common oak. Additionally, passive biomonitoring with trees has the disadvantage that effects of soil characteristics (e.g., nutrient availability) cannot be accounted for. Nutrient availability can influence SLA by changing lamina and mesophyll thickness (Meziane and Shipley 1999). Moreover, no information was available on other (a) biotic stressors that may have occurred in the past, such as historic management (e.g., pruning intensity), (mechanical) soil disturbances and herbivore attacks and diseases. Mechanical soil disturbances can, for example, increase plant FAA (Freeman et al. 2005). Her-

bivory can cause changes in the microclimate of the remaining foliage and increase the specific hydraulic conductance of the damaged leaves, leading to an increased stomatal conductance (Pataki et al. 1998). To avoid these confounding effects of passive biomonitoring, active biomonitoring (Chapter 3-6), i.e., with organisms that are introduced in the ecosystem, can be performed instead.

Not only the presence of other environmental stressors, but also the tolerance of common oak for $NH_3$ can help to explain the absence of adverse effects on the considered leaf characteristics. The sensitivity of different plant species to $NH_3$ exposure is listed by Krupa (2003), with common oak as intermediate susceptible for short-term exposures to high $NH_3$ concentrations. The sensitivity of oak to lower $NH_3$ concentrations over longer periods is not known. The high N availability in the soil due to high $NH_x$ deposition near intensive livestock farms might increase the $NH_4^+$ pools and apoplastic pH in leaf tissue, causing an increased stomatal compensation point (Mattson and Schjoerring 2002) and, therefore, a lower direct absorption of potentially harmful $NH_3$. It is also possible that the measured leaf characteristics are not sensitive to the ambient $NH_3$ concentration, since leaf characteristics of a same tree can respond differently to ambient air pollution (Chapter 3). Therefore, more biochemical and/or physiological leaf characteristics should be measured, such as ascorbate, glutathione, superoxide dismutase and chlorophyll fluorescence -as they might reflect changes that cannot be detected at the anatomical or morphological level- before the suitability of a species as biomonitor can be correctly assessed.

## 2.5 Conclusions

Our results indicated that specific leaf area, fluctuating asymmetry, relative chlorophyll content and stomatal resistance of common oak are not good biomonitors for monitoring four-monthly mean atmospheric $NH_3$ concentrations in the vicinity of livestock farms. Moreover, these leaf characteristics demonstrate a high within-plant and between-plant variability, which reflects a high leaf sensitivity and questions the effectiveness of common oak as a passive biomonitor. The lack of relationships between the studied leaf characteristics and the mean four-monthly $NH_3$ concentration can be caused by confounding effects of (i) other environmental factors, (ii) genetic differences, (iii) tree history in relation to human and natural disturbances and (iv) intermediate susceptibility of common oak, due to a possibly high stomatal compensation point for $NH_3$. Therefore, we conclude that the use of an active biomonitor is more appropriate than the use of a

passive biomonitor, and that the measurement of other environmental factors, such as $O_3$, $SO_2$, $NO_x$ and air temperature are necessary when performing a biomonitoring study. The use of an active biomonitor reduces the variability caused by genotypes and soil characteristics and, therefore, the effectivity of several species as active biomonitor needs to be tested. In general, a lot of research is still necessary to evaluate the potential of trees as active or passive biomonitors.

# 3

# The potential of biomonitoring of air quality by using leaf characteristics of white willow

*After: Wuytack, T., Verheyen, K., Wuyts, K., Kardel, F., Adriaenssens, S., Samson, R., 2010. The potential of biomonitoring of air quality using leaf characteristics of white willow (Salix alba L). Environmental Monitoring and Assessment, 171, 197-204.*

In this study, we assess the potential of white willow as biomonitor for monitoring the ambient air quality. Therefore, shoot biomass, specific leaf area (SLA), stomatal density, stomatal pore surface (SPS) and stomatal resistance were assessed from leaves of stem cuttings. The stem cuttings were introduced in two regions in Belgium with a relatively high and a relatively low level of air pollution, i.e., Antwerp city and Zoersel respectively. In each of these regions, nine sampling points were selected. At each sampling point, three stem cuttings of white willow were planted in potting soil. Shoot biomass and SLA were not significantly different between Antwerp city and Zoersel. Microclimatic differences between the sampling points may have been more important to plant growth than dif-

ferences in air quality. However, SPS and stomatal resistance of white willow were significantly different between Zoersel and Antwerp city. The SPS was 20% lower in Antwerp city due to a significant reduction in both stomatal length (-11%) and stomatal width (-14%). Stomatal resistance at the adaxial leaf surface was 17% higher in Antwerp city because of the reduction in stomatal pore surface. Based on these results, we conclude that stomatal characteristics of white willow are potentially useful indicators for air quality.

## 3.1 Introduction

Air pollutants has been abundantly associated with many adverse ecological effects, such as vitality losses, decreasing species diversity and shifts in community composition (Spellerberg 1998, see §1.2.2). To protect vegetation, air quality limit values were established for the most important pollutants (see §1.1.3) and concentrations of these pollutants are measured by air quality monitoring stations using physico-chemical methods. These methods provide information about the concentration of a single pollutant and not about their cumulative, antagonistic or synergistic effects and do not take into account the influence of meteorological conditions on the effect of air pollutants on vegetation (Fuhrer et al. 1997). Consequently, a rising interest in biomonitoring (see §1.3), which gives a more realistic assessment of the impact of air quality on ecosystems (Falla et al. 2000), is observed. As plants are immobile and more sensitive in terms of physiological reaction to the common air pollutants than humans and animals, they better reflect local conditions (Raz et al. 2011). Biomonitoring can be performed through analyses on the vegetation already present in a given study area (so-called passive biomonitoring, see Chapter 2), or carried out with selected test plants introduced at the study site (active biomonitoring) (Nali and Lorenzini 2007, see §1.3.1). Several active biomonitoring studies have been carried out under controlled circumstances in open top chambers or greenhouses to investigate the effect of various (mixtures of) air pollutants on plants (e.g., Broadmeadow 2000, Monaci et al. 2000, Novak 2003). Only a few active biomonitoringstudies have been performed under field conditions (Calzoni et al. 2007, Franzaring et al. 2007, Rey-Asensio and Carballeira 2007). Therefore, the main objective of this exploratory study was to evaluate the potential of white willow as an active biomonitor of ambient air quality (urban versus rural). Since the assimilative organs of the plants are the most directly affected organs of polluted air, this chapter focuses on leaf characteristics and we hypothesized that stomatal charac-

teristics and specific leaf area (SLA) will be adapted by the ambient air pollution.

## 3.2 Materials and methods

### 3.2.1 Study area

Two experimental sites were selected in the north of Flanders, i.e., Antwerp city (51° 13' N, 4° 24' E) and Zoersel at 20 km north-east of Antwerp city (51° 16' N, 4° 42' E) (Fig. 3.1). The city of Antwerp is a densely populated urban area (466 203 inhabitants on 2 300 $km^2$) with a high industrial activity in the harbor (mainly petrochemical). The municipality of Zoersel, in contrast, is a moderately but increasingly populated rural area (20 803 inhabitants on 543 $km^2$) with a relatively high forest cover, some agricultural activities and no industrial activity. Air quality measuring stations of the Flemish Environmental Agency (VMM) (Fig. 3.1) in Hoboken and Borgerhout provided data of the air pollutant concentrations ($O_3$, $NO_2$, NO, $SO_2$ and $PM_{10}$) in Antwerp city, while data from the measuring stations in Schoten, Schilde and Beerse were used as an indication for the air quality in Zoersel. Meteorological data was obtained from the weather stations (KMI).

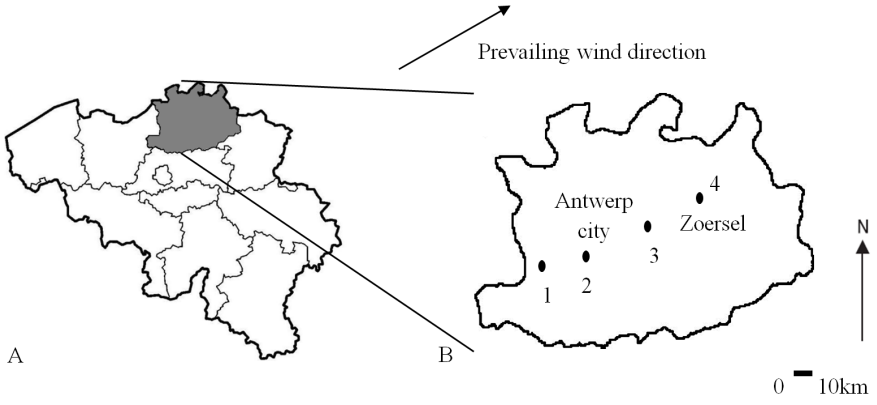


Figure 3.1: Location of the experimental sites (b) where white willow were transplanted in the north of Belgium (a), i.e. Antwerp city (polluted) and Zoersel (less polluted); the black dots (b) indicate the measuring stations of the Flemish Environment Agency, i.e., Hoboken (1), Borgerhout (2), Schilde (3) and Beerse (4)

### 3.2.2 Experimental design

At each of the two experimental sites, nine sampling locations were randomly selected in private gardens. In each of these sampling locations, three stem cuttings of white willow were planted in pots with homogeneous potting soil (pH-$H_2O$ 5-6) in April '07. The stem cuttings were provided by De Vos 'Salix', a company specialized in the cultivation and processing of *Salix* sp. White willow was chosen as biomonitor because of (i) the high gas exchange rate typical for fast growing species, like white willow (Novak et al. 2003), and hence higher interaction with atmospheric gases (Rennenberg et al. 1996) and (ii) the availability of genetically identical stem cuttings excluding genetic variability.

Overgrowth by competitive vegetation, snail herbivory and water deficiency reduced the original number of sampling locations. In Antwerp city and Zoersel, six and eight of the nine sampling locations remained, respectively. In total, fourteen stem cuttings developed in Antwerp city, with minimum two and maximum three stem cuttings per sampling location, and seventeen in Zoersel, with minimum one and maximum three stem cuttings per sampling location. Plant shoots, here defined as a leaved branch of a stem cutting, were harvested five months after planting, in September '07. In Antwerp city, twenty-eight shoots developed, while in Zoersel, thirty-four shoots were available in September '07.

### 3.2.3 Data collection

#### 3.2.3.1 Biomass variables

Following harvest, total leaf area ($cm^2$) of all leaves on a shoot was determined by scanning of the leaves with a leaf area meter (Li-300 leaf area meter, Li-COR, Lincoln, Nebraska, accuracy $0.01 cm^2$). Leaves that were not immediately scanned after harvest were stored in a cool box and scanned within 24 hours. Dividing the total leaf area by the number of leaves on a shoot yielded the mean leaf area (MLA, $cm^2$). Leaves and shoots were subsequently dried during 48 h at 70°C and weighted to obtain total shoot biomass per stem cutting. Shoot biomass (SB, g) was calculated as the total shoot biomass divided by the number of shoots for each stem cutting. For calculating the mean leaf biomass (MLB, g), the total leaf biomass of a shoot was divided by the number of leaves on that shoot. SLA was calculated according to Eq. 2.1 (Chapter 2).

#### 3.2.3.2 Stomatal characteristics

Willow has amphistomatous leaves, showing stomata on both the adaxial and abaxial leaf side. Therefore, stomatal imprints at both abaxial and adaxial leaf sides of three developed, healthy, leaves of each shoot were made prior to harvesting according to the method described in §2.2.2.3. In addition, stomatal density (SD), stomatal pore surface (SPS) were measured and stomatal resistance ($R_S$) was calculated by using Eq. 2.3 (Chapter 2).

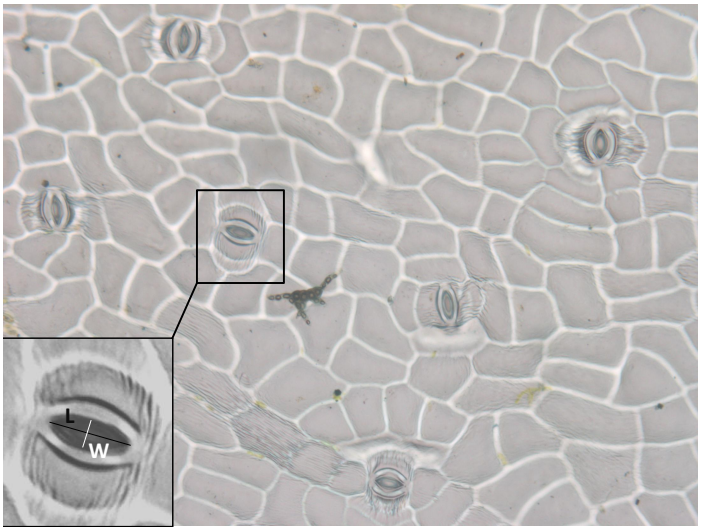


Figure 3.2: Image of stomata on the adaxial leaf side of white willow with indication of the stomatal length (L, µm) and width (W, µm)

The abaxial leaf side of white willow was densely covered with trichomes, which made it impossible to determine the stomatal characteristics on this side. Plucking the hairs of the leaves caused a deformation of the cells and stomata. For this reason, only the adaxial side was taken into account for measurements and statistical analysis.

#### 3.2.3.3 Herbivory as an external confounding factor

The influence of (snail) herbivory, on growth and shoot and leaf morphology was taken into account. Therefore, a scale of herbivore damage was developed (0: no herbivore damage; 1: minimal herbivore damage; 2: moderate herbivore damage; 3: extreme herbivore damage).

### 3.2.4 Statistical analysis

The dataset was tested for normality. If data were not normally distributed ($p < 0.05$), a log transformation was performed. Differences in plant parameters between sampling locations and the difference between the experimental sites were analyzed using a nested general linear model, with experimental site as fixed factor (Antwerp city, Zoersel), sampling location as a random factor nested within site and the external confounding factor as covariate. All statistical tests were performed with SPSS 15.0.

## 3.3 Results

### 3.3.1 Air quality and meteorology

During the study period (April 2007 - September 2007) the NO and $NO_2$-concentrations were higher in Antwerp city than in Zoersel, in contrast with the $O_3$ concentration (Table 3.1). The maximum hourly value for $O_3$ was during the study period 159 μg $m^{-3}$ in Antwerp city and 175 μg $m^{-3}$ in Zoersel. During the months April, August and September the $SO_2$ concentration was higher in Antwerp city than in Zoersel (results not shown), but the mean $SO_2$ concentration in Antwerp city (Hoboken) was during the study period comparable with the mean $SO_2$ concentration in Zoersel (Table 3.1).

Table 3.1: Mean concentration (μg $m^{-3}$) ± standard deviation of $NO_2$, $O_3$ and $SO_2$ during the study period (April - September 2007) and AOT40 (μg $m^{-3}$ hours) for Antwerp city and Zoersel. The p-value of the independent t-test is also given; the p-value followed by a '*' indicates a significant difference at the 0.05 level.

| | $NO_2$ | $O_3$ | $SO_2$ | $PM_{10}$ | AOT40 |
|---|---|---|---|---|---|
| Antwerp | 33.5 ± 11.3 | 35.8 ± 19.2 | 10.4 ± 7.1 | 30.5 ± 14.3 | 3518 |
| Zoersel | 28.0 ± 9.9 | 46.7 ± 17.9 | 10.3 ± 8.2 | 23.2 ± 12.3 | 6359 |
| p-value | < 0.000* | < 0.000* | 0.962 | < 0.000* | / |

In 2007, average air temperature and relative humidity was 11.5°C and 80% respectively and annual rainfall amounted to 880 mm. The warmest month was June (on average 17.5°C) and the coldest month was December (on average 4.1°C). During the study period (April 2007 - September 2007), average air temperature was 15.8°C and total rainfall amounted to 414 mm (www.kmi.be). However, exceptionally, the month of April was completely rainless.

### 3.3.2 Biomass variables

Table 3.2 gives an overview of mean MLA, MLB, SLA and SB in Antwerp city and Zoersel. The MLA, MLB and SB were not significantly different between Antwerp city and Zoersel and between the sampling locations. On the contrary, SLA was significantly different between the sampling locations, but not between Antwerp city and Zoersel.

Table 3.2: Mean ± standard deviation of mean leaf area (MLA, $cm^2$), mean leaf biomass (MLB, g), specific leaf area (SLA, $cm^2\ g^{-1}$) and shoot biomass (SB, g) in Antwerp city and Zoersel

| | Antwerp city | Zoersel |
|---|---|---|
| MLA | 3.76 ± 2.63 | 3.91 ± 1.83 |
| MLB | 0.027 ± 0.0018 | 0.028 ± 0.013 |
| SLA | 143.6 ± 26.9 | 143.1 ± 35.6 |
| SB | 1.30 ± 1.38 | 1.87 ± 3.50 |

### 3.3.3 Stomatal characteristics

Mean values of all studied stomatal characteristics and their minimum and maximum values and standard deviation are given in Table 3.3 for Antwerp city and Zoersel individually.

Table 3.3: Mean ± standard deviation of stomatal density (SD, $mm^{-2}$), stomatal pore surface (SPS, $\mu m^2$), stomatal length (L, μm), stomatal width (W, μm) and stomatal resistance ($R_S$, s $m^{-1}$) of white willow, sampled at each of the experimental site, Antwerp city and Zoersel, n the number of sampling locations. The p-value followed by a '*' indicates a significant difference at the 0.05 level.

| | Antwerp city | Zoersel | n | F-value | p-value |
|---|---|---|---|---|---|
| SD | 85.7 ± 15.4 | 78.5 ± 11.5 | 31 | 0.004 | 0.852 |
| SPS | 66.2 ± 10.4 | 87.1 ± 14.6 | 30 | 12.3 | 0.005* |
| L | 13.9 ± 0.9 | 15.7 ± 1.7 | 30 | 7.7 | 0.001* |
| W | 6.1 ± 0.7 | 7 ± 0.7 | 30 | 9.7 | 0.011* |
| $R_S$ | 109.6 ± 27.1 | 91.1 ± 17.3 | 29 | 4.1 | 0.033* |

Figure 3.3a shows that the SD in Antwerp city was higher than in Zoersel by 9%, but the difference was not significant (Table 3.3). As illustrated in Fig. 3.3b, the mean SPS in Antwerp city was significantly lower (-20%) in comparison with the mean SPS in Zoersel. This decrease in SPS at Antwerp

city in comparison with Zoersel was due to a significant reduction in stomatal length (L) and width (W), with 11.4% and 14.1% respectively. Since SPS was reduced more than SD was increased, $R_S$ was found to be significantly higher at Antwerp city than in Zoersel (Fig. 3.3c). In total, an increase in $R_S$ of 17% was observed between Antwerp city and Zoersel. The stomatal characteristics were not significantly different between the sampling locations of each experimental site.

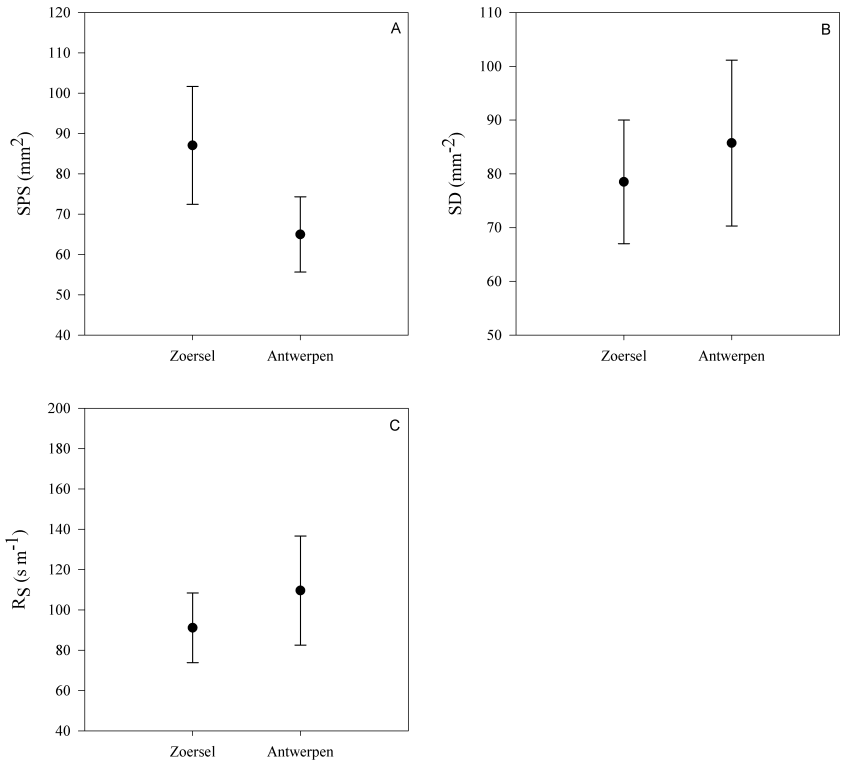


Figure 3.3: Error bars of a) average adaxial stomatal pore surface (SPS), b) average adaxial stomatal density (SD) and c) average adaxial stomatal resistance ($R_s$) of white willows in Antwerp city and Zoersel. Error bars indicate 95% confidence interval

Herbivory had no significant influence on any of the biomass and stomatal characteristics (minimal p-value = 0.304).

## 3.4 Discussion

### 3.4.1 Active biomonitoring with white willow

Previous research showed phytotoxic effects of air pollutants (i.e., Moraes et al. 2002, Larcher 2003). However, many of these biomonitoring studies looked solely at the individual pollutants and were performed under controlled conditions (Honour et al. 2009). In this way the advantage of biomonitoring, namely that the effect of a constant varying mixture of air pollutants on plants can be determined in real-life, is ignored. In addition, active biomonitoring has also several advantages over passive biomonitoring. Firstly, active biomonitors can be planted in the same uniform substrate thereby excluding possible confounding effects like, e.g., soil nutrient availability. Secondly, the selection of the sampling locations is not dependent on the occurrence of the studied biomonitors, as is the case with passive monitoring. Thirdly, genetic variability, which may be linked with pollution tolerance, can be better controlled in active monitoring (Larcher 2003). Finally, age dependent differences in response to air pollution as can occur in passive biomonitoring can be avoided (Dobbertin 2005). For this reason, white willow was planted as an active biomonitor to determine the impact of the ambient air quality on plant characteristics.

However, during our study, we encountered several problems with water supply, e.g., the exceptional dry month of April, and snail herbivory. White willow seems to be highly sensitive to these problems. In following experiments these factors should be controlled by keeping the pots well watered as, e.g. done by Mills et al. (2007), or protecting against snail herbivory by mechanical means like copper bands wrapped around the pots. When planting the willows in private gardens, rules on water supply, chemical treatments etc. should be strict and standardized. In public parks, care should be taken in terms of overgrowth by other vegetation and vandalism.

### 3.4.2 Biomass variables

The adjustment of biomass, caused by a changing allocation of carbohydrates, is the final process of different internal and external processes due to air pollution. According to Fuhrer et al. (1997), this final process can only be detected on the long term (more than one year). Probably, for this reason, no significant differences of MLB and SB between Antwerp city and Zoersel were detected. Also, the significant differences between the sampling locations, indicates that shoot biomass mainly changed due to differences in local microclimate.

The impact of air pollution on MLA and SLA is widely discussed in literature. The results are not univocal, probably because of the species-specific responses. According to Broadmeadow and Jackson (2000) a high $O_3$ concentration reduces SLA, while Balasooriya et al. (2009) and Carreras et al. (1996) reported an increase in SLA of *Taraxacum officinale* and *Ligustrum* species, respectively, in more polluted areas. In this study, SLA of white willow was not significantly influenced by the ambient air quality, but also the local microclimate at each sampling location seems to have influenced SLA. Micro-environmental variation occurs because each plant and each leaf of a plant is exposed to a different combination of environmental factors (Cowart and Graham 1999). Ontogenetic variation occurs because leaves may differ in their stage of development. Poorter et al. (2009) demonstrated a twofold difference of SLA within a single plant, due to a gradient in light, air temperature, air humidity and wind velocity and plant specific factors (Gunn et al. 1999, Barber et al. 2004), such as age, development stage and position of the leaf on the plant and genetics. In addition, not only SLA can be influenced by the microclimate, but several other leaf characteristics, such as fluctuating asymmetry (FA), leaf wettability and $R_S$ can be influenced by the microclimate. For example, Møller and Swaddle (1997) stated that FA reveals aspects of individual tree quality, rather than aspects of population quality, and Bagchi et al. (1989) showed that leaves of teak growing in the middle of the crown were more symmetrical than those higher or lower on the plant. The environmental conditions under which a leaf develops can also lead to pronounced differences in $R_S$ between individual leaves of a single tree: leaves produced under warmer air temperatures have lower SD compared to leaves produced under colder air temperatures (Beerling and Chaloner 1993). Koch et al. (2006) stated that the chemical composition of the epicuticular wax layer shows indeed a great variability among different plant species, different organs of an individual plant or during the ontogeny of individual organs.

### 3.4.3 Stomatal characteristics

Modifications leading to an optimal adjustment for controlling gas exchange in general and the entrance of pollutants through stomata in particular can originate in two ways (Rashidi et al. 2012). Plants may reduce their pollutant uptake by simply decreasing their SD or there may be an increase in SD and a concomitant reduction in SPS (Elagoz et al. 2006, Verma and Singh 2006, Balasooriya et al. 2009, Rashidi et al. 2012). Our findings are consistent with the latter scenario, since SD was increased (although not significantly) by 8.4% and SPS was significantly decreased by 24% in

Antwerp city, compared to Zoersel. The formation of more but smaller stomata in Antwerp city can be seen as a measure to minimalise the uptake of pollutants whilst optimising the $CO_2$ uptake and reducing the loss of water due to transpiration (Balasooriya et al. 2009).

Changes in SD and SPS have an opposite influence on $R_S$ (Elagoz et al. 2006). The $R_S$ expresses the extent of the inhibition of gas diffusion through stomata. In case of air pollution stress, limitation of gas diffusion is observed due to an increase of $R_S$ (Balasooriya et al. 2009, Verma and Singh 2006). Our results confirm these findings, since in Antwerp city, the decrease in SPS was larger than the increase in SD, causing a net increase in $R_S$ by 17%.

## 3.5 Conclusions

The availability of genetically identical stem cuttings and its high gas exchange rate are potential advantages of white willow for air quality monitoring. The results indicate that white willow growing in more polluted environments adapts by forming more but smaller stomata, causing a net increase in $R_S$. Hence, we conclude that stomatal characteristics of white willow are potentially good biomonitors for monitoring the air quality. When white willow is applied as an active biomonitor, several aspects need to be considered: (i) sufficient water supply should be provided, (ii) attempts should be made to minimize herbivory and (iii) good arrangements with the land-owners need to be made so that weeding, water supply, chemical treatments etc. is done in a standardized way.

# 4

# The effect of ambient air quality on leaf characteristics of white willow during two consecutive years

*After: Wuytack, T., Samson, R., Van Wittenberghe, S., Wuyts, K., Verheyen, K., 2012. The response of leaf characteristics of white willow (Salix alba L.) to ambient air pollution during two consecutive years. Submitted to Environmental and Experimental Botany.*
*After: Wuytack, T., Wuyts, K., Van Dongen, S., Baeten, L., Kardel, F., Verheyen, K., Samson, R., 2011. The effect of air pollution and other environmental stressors on leaf fluctuating asymmetry and specific leaf area of Salix alba L.. Environmental Pollution 159, 2405-2411.*

White willow was exposed to variable levels of ambient air pollution for two consecutive years. We investigated how air quality affects specific leaf area (SLA), stomatal characteristics, leaf area fluctuating asymmetry (FAA), leaf wettability and chlorophyll fluorescence and how these responses depend on exposure time, taking into account other environmental factors. Cuttings were grown in standardized conditions in the near vicinity of air quality monitoring stations in Bel-

gium. With the exception of stomatal pore surface (SPS), the response of leaf traits to ambient air pollution was not different between the first and second in-leaf season. The SPS was influenced by the ambient air quality during the second in-leaf season, which caused a change in the stomatal resistance ($R_S$) as an adaptation to the long-term exposure to air pollution. During both in-leaf seasons, SLA increased with increased shade; after reducing the confounding effect of shade, SLA also correlated with the mean $NO_2$ and $O_3$ concentrations. Leaf wettability and chlorophyll fluorescence were only slightly influenced by shade, while the variation of FAA could not be explained by any of the environmental factors considered in this study. In conclusion, SLA and $R_S$ can be used to monitor the ambient air pollution, when similar degrees of shade are taken into account.

## 4.1 Introduction

Plant characteristics, mainly traditional fitness components such as growth and total biomass production, are frequently used in air quality studies (Woodbury and Laurence 1994, Sant'Anna-Santos et al. 2006). For example, Lovett et al. (2009) showed that $O_3$ affects the cell membrane functioning, leading to reduction in photosynthesis and thus slower tree growth. Unfortunately, growth and biomass production determination is destructive and time-consuming. Instead, morphological (e.g., fluctuating asymmetry (FA), specific leaf area (SLA)), physiological (e.g., chlorophyll fluorescence, stomatal resistance ($R_S$)) , anatomical (e.g., leaf wettability) and biochemical (e.g., chlorophyll content, malondialdehyde) leaf characteristics can be used as rapid, non-destructive, diagnostic monitoring tools.

Physiology of plants is influenced by air pollution through a depression of the photosynthetic performance, caused by a reduced ability to channel solar energy through the photochemical pathways or due to a degradation of chlorophyll. It has also been proven that the photosynthetic performance is related to leaf wettability (Brewer and Smith 1995) and stomatal conductance (Schenone et al. 1994, Larcher 2003). Leaf wettability can be changed by air pollution due to a changed quantity, chemical composition and/or structure of the epicuticular wax layer (Percy et al. 1992). Since photosynthetic performance and chlorophyll fluorescence are in balance, a decrease in photosynthetic performance will lead to an increase in chlorophyll fluorescence. Bortier et al. (2000) and Flowers et al. (2007)

showed an increased chlorophyll fluorescence of beech and snap bean under elevated $O_3$ concentrations, hereby confirming that $O_3$ decreased the photosynthetic capacity and the efficiency of excitation capture. In addition, a disruption of the photosynthetic performance indicates that air pollution stress is energy-dissipative. Consequently, less energy is available for preserving homeostasis, leading to errors in development (Graham et al. 2003). Fluctuating asymmetry (FA) has been used to estimate this developmental noise in morphological traits (Palmer and Strobeck 1986, Hao and Xiangrong 2006) and is proved to be a reliable indicator of a wide variety of biotic and abiotic stresses, such as defoliation (Otronen and Rosenlund 2001), habitat quality (Velickovic and Perisic 2006) and salinization (Roy and Stanton 1999). However, several studies report just as well no changes in asymmetry in stressful conditions, such as air pollution (Kozlov et al. 2009), salinization (Sinclair and Hoffmann 2003), metal pollution (Ambo-Rappe et al. 2008) and nutrient deficiency (Black-Samuelsson and Andersson 2003).

Not only developmental noise in morphological traits, but also the morphological traits itself, such as leaf area, thickness and density can be influenced by air pollution (Dineva 2004, Dobbertin 2005), indicating a metabolic investment to avoid or at least to compensate cellular damage (Dineva 2004). Leaves with a low density and thus a large volume of intercellular spaces, are characterized by a high leaf conductivity, which in turn, may facilitate photosynthesis (Poorter et al. 2009 and references herein). $O_3$ sensitive species are characterized by a low leaf density, which is associated with a high gas exchange capacity and thus with a high uptake of $O_3$ (Gravano et al. 2003). A high leaf density can be caused by a large fraction of mesophyll cells or a high proportion of lignified tissues, important for leaf toughness, and thereby leaf and plant survival (Poorter et al. 2009 and references herein). Thick leaves allows a higher concentration of photosynthetic apparatus per unit leaf area, while thinner, but larger leaves, allows a higher light interception (White and Montes 2005). Since the determination of leaf thickness and density is not straightforward, a relative measure is used, namely, SLA (Larcher 2003). SLA of most terrestrial species ranges between 30 and 330 $cm^2\ g^{-1}$ (Poorter et al. 2009), indicating a high variability in SLA. For example, fast-growing species develop more leaf area per unit leaf biomass, leading to a higher growth rate (Poorter and van der Werf 1998). Shrubland, desert and woodland species have an extreme low SLA, since either drought, nutrient limitation or both hamper growth (Poorter et al. 2009). Also, air pollution can lead to a high variability in SLA; SLA of *Ligustrum lucidum* and *Leontodon helveticus* increased as a

consequence of $SO_2$ pollution from fuel combustion and $O_3$ pollution, respectively, for compensating the inhibition of photosynthesis (Bassin et al. 2009). However, SLA of carrot plants decreased due to a decreased leaf production rate caused by air pollution (Tiwari et al. 2006) and, in general, SLA decreases as a consequence of elevated $CO_2$ concentrations (Poorter et al. 2009).

A large part of the biomonitoring studies, mentioned above, are performed in the vicinity of point polluters where a (extreme) high concentration of air pollutants is present. Consequently, these studies do not provide information about the effect of common air pollutant concentrations on plants, as observed in densely populated and urbanized areas. In addition, plant responses to air pollution can also vary over time, which highlights the importance of taking the exposure time to air pollutants into account. Several studies have investigated the effect of exposure time to $NH_3$ (van Hove et al. 1989, Munzi et al. 2010), $SO_2$ (Tomassini et al. 1977), $O_3$ (Beyers 1992, Fincher and Alscher 1992, Talhelm et al. 2012) and industrial pollution (Eranen and Kozlov 2006) on the response of plant characteristics. However, most of these studies investigated the effect of a relatively short exposure time of a few days, weeks or months, or they did not investigate the effect of air pollutants under field conditions. Therefore, the goal of this study was to find a time- and cost-effective biological indicator, sensitive to common air pollutant concentrations, for supporting the traditional physico-chemical approach in air quality assessments, and to test whether exposure time plays a role in the response of this indicator. We hypothesized that (i) SLA, stomatal characteristics, leaf wettability, leaf area fluctuating asymmetry (FAA) and/or maximum photochemical efficiency of photosystem II ($F_v/F_m$) would adapt to the ambient air quality and (ii) that the cumulative effect of two consecutive years of air pollution exposure would be reflected in an enhanced response of these leaf characteristics compared to one-year exposure.

## 4.2 Materials and methods

### 4.2.1 Study area

White willow cuttings were grown in the near vicinity of air quality monitoring stations in Belgium (see §1.1.3.2). The stations are operated by the Flemish Environmental Agency (VMM), the Walloon 'Institut Scientifique de Service Public' (ISSeP) and the Brussels Institute for Management of the Environment (IBGE-BIM) and classified in urban, suburban, rural and

industrial (Fig. 1.3, Table 4.1, www.irceline.be).

Table 4.1: Location (longitute and latitude) of each monitoring station and corresponding land use class

| Station | Longitude | Latitude | Class |
|---|---|---|---|
| Aarschot | 50°58'39" | 4°50'15" | Rural |
| Berendrecht | 51°20'56" | 4°20'23" | Industrial |
| Bergen | 50°27'55" | 3°56'20" | Suburban |
| Borgerhout | 51°12'34" | 4°25'54" | Urban |
| Charleroi | 50°25'44" | 4°27'31" | Urban |
| Corroy | 50°39'20" | 4°40'07" | Rural |
| Dourbes | 50°05'45" | 4°35'41" | Rural |
| Engis | 50°34'60" | 5°23'51" | Suburban |
| Hasselt | 50°56'23" | 5°22'06" | Suburban |
| Mendonk | 51°09'00"' | 3°48'31" | Industrial |
| Schoten | 51°15'08" | 4°29'30" | Suburban |
| Sinsin | 50°16'24" | 5°14'04" | Rural |
| Ukkel | 50°47'51" | 4°21'33" | Urban |
| Veurne | 51°00'59" | 2°34'55" | Rural |
| Voorhaven | 50°53'01" | 4°22'59" | Industrial |
| Zwevegem | 50°48'54" | 3°19'21" | Suburban |

### 4.2.2 Experimental design

White willow was chosen as an active biomonitor because of good experiences in a previous study (Chapter 3). In April 2009, twelve stem cuttings of white willow (length: 18cm) were placed at each monitoring station. Due to practical reasons (lack of space at each monitoring station), it was impossible to plant more than twelve stem cuttings. However, comparable, or even smaller, sampling sizes of trees were found in literature (Otronen and Rosenlund 2001, Hodar 2002). Each stem cutting was planted in 3.5 $dm^3$ pots with homogeneous potting soil (pH-$H_2O$ 5.5) and 2/3 of each cutting was buried below pot soil level. Since the rooting volumes were not 'fenced off' with a porous membrane, root expansion outside the pots could occur through the holes in the bottom. At planting, no cutting had leaves, shoots, or roots. Plants were spaced, to minimize shading. To avoid water deficiency, a semi-automatic, capillarity-based water supply system was used. The water supply system consisted of a water reservoir connected with the potting soil through glass fiber ropes (5 mm diame-

ter) (Fig. 4.1). The water reservoirs were shut off from incoming rainfall and were refilled monthly with tap water. To counteract snail herbivory, copper tape was attached around the pots. Plants with damage from insect herbivory, mainly by caterpillars, were not treated with insecticides. In September 2009, willows were harvested and stomatal characteristics, $F_v/F_m$, leaf wettability, FAA and SLA were determined. Due to mortality, which is a side effect of working with living materials in real conditions, less than twelve willows remained by the time of harvesting at some monitoring stations (ten in 'Charleroi', eleven in 'Hasselt', seven in 'Schoten', and six in 'Voorhaven').

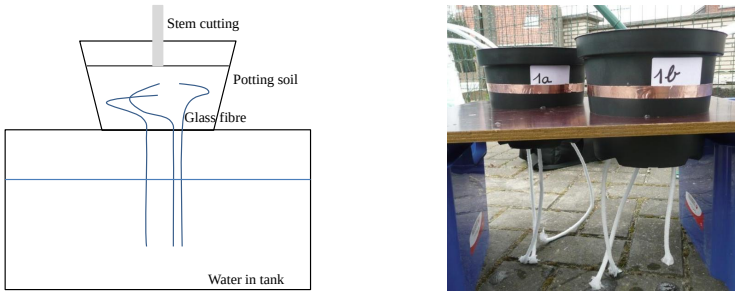


Figure 4.1: Schematic overview of the semi-automatic water supply system

In April 2010, four of the best-performing willows (based on the growth information obtained from the in-leaf season) were selected at each monitoring station for the second sampling period. To prevent nutrient depletion of the soil, 20 ml of an inorganic fertilizer (3% N-$NH_4$ and N-$NH_3$, 2% $P_2O_5$, 5% $K_2O$) was added once to each pot. In September 2010, these willows were harvested for the second time and the same leaf characteristics as in the first in-leaf season were measured. In September 2011, we planned a third sampling period, but all of the willows showed reduced growth and produced deformed and yellowish leaves. Therefore, the data from the third in-leaf season were not used for further analysis.

### 4.2.3 Data acquisition

#### 4.2.3.1 Air quality data

Since mainly $SO_2$, NO, $NO_2$, $O_3$ and $PM_{10}$ are toxic for plants (Fuhrer and Bungener 1999), concentrations ($\mu g\ m^{-3}$) of those air pollutants, averaged over the first and second in-leaf season, were used to describe the ambient

air quality at each monitoring station (Table 4.2). In addition, a site specific value for air quality was obtained by using a Principal Component Analysis followed by a Varimax rotation with Kaiser Normalization.

#### 4.2.3.2 Fluctuating asymmetry and specific leaf area

At each monitoring station, we collected randomly 20 fully developed and undamaged leaves to calculate FAA and SLA for the first and second in-leaf season. SLA and FAA were measured and calculated according to the method described in §2.2.2.2 and preliminary analyses of FAA were performed as described in §2.2.3.

For illustration and interpretation purposes, leaf cross-sections were taken from eight randomly selected leaves at all monitoring stations in September 2010. One leaf disc was punched out per leaf, excluding the mid vein, and stored in a fixation solution (formaldehyde, 10 ml formal 37%, 50 ml ethanol 96%, 5 ml acidic acid and 35 ml distilled water). Samples were dehydrated through a graded alcohol series (50% - 70% - 80% - 96% isopropanol) and first embedded in a mixture of paraffin and chloroform (1:1). After several hours, samples were embedded in paraffin and, thereafter, slides (thickness 20 μm) were made with a rotation microtome and stained with Astra blue.

#### 4.2.3.3 Stomatal characteristics

For the first and second in-leaf season, stomatal imprints were taken from the adaxial side of ten fully developed, healthy leaves prior to harvesting according to the method described in Chapter 2 (see §2.2.2.3). Stomatal imprints were used to measure stomatal density (SD), stomatal pore surface (SPS) and to calculate $R_S$ by using Eq. 2.3 (Chapter 2).

Table 4.2: Shade (%), mean atmospheric concentration (μg $m^{-3}$) of $SO_2$, NO, $NO_2$, $O_3$ and $PM_{10}$, mean air temperature (°C) and relative air humidity (%) during the first (April - September 2009) and second (April - September 2010) in-leaf season; the AOT40 (μg $m^{-3}$ hours) is given between parentheses.

| | | First in-leaf season | | | | | | | Second in-leaf season | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Station | Shade | $SO_2$ | NO | $NO_2$ | $O_3$ (AOT40) | $PM_{10}$ | T | RH | $SO_2$ | NO | $NO_2$ | $O_3$ (AOT40) | $PM_{10}$ | T | RH |
| Aarschot | 30 | 1.7 | 2.4 | 15.9 | 57.9 (19997) | 15.2 | 15.9 | 69.3 | 1.8 | 2.6 | 16.2 | 60.9 (19000) | 22.4 | 15.4 | 72.6 |
| Berendrecht | 37 | 6.1 | 7.8 | 26.7 | 48.5 | 32.1 | 16.4 | 70.5 | 3.5 | 10.6 | 28.2 | 42.9 | 28.2 | 15.6 | 70.3 |
| Bergen | 40 | 0.4 | 8.4 | 25.1 | 41.6 | 22.4 | 15.5 | 71.6 | 0.7 | 7.8 | 26.3 | 39.4 | 23.2 | 15 | 69.1 |
| Borgerhout | 65 | 4.4 | 8.7 | 37.9 | 43.1 | 28.7 | 16.7 | 64.8 | 3.4 | 9.4 | 37.2 | 41.6 | 24.7 | 16.1 | 63.6 |
| Charleroi | 40 | 0.8 | 4.8 | 23.6 | 48 | 21.2 | 15.6 | 71.1 | 1.9 | 5.6 | 25.3 | 49.6 | 23.5 | 15.9 | 67.5 |
| Corroy | 10 | 0.8 | 2.4 | 16.4 | 58.1 | 20.8 | 15.1 | 82.6 | 0.8 | 4.1 | 20.4 | 55.8 | 21.9 | 14.4 | 83.5 |
| Dourbes | 24 | 1.0 | 0.9 | 4.0 | 65.4 (18980) | 14.6 | 14.5 | 83.8 | 2.2 | 1.3 | 5.9 | 65 | 18.2 | 13.9 | 83.8 |
| Engis | 22 | 5.7 | 2.1 | 18.3 | 57.4 | 25.9 | 16.6 | 68.3 | 5.1 | 2.8 | 20 | 57 | 25.9 | 16.4 | 64.6 |
| Hasselt | 23 | 1.6 | 4.5 | 21.2 | 56.2 (21674) | 18 | 14.7 | 68.8 | 1.8 | 5.3 | 22.6 | 57.4 (20000) | 20.9 | 14.5 | 67.6 |
| Mendonk | 37 | 3.6 | 3.5 | 22.0 | 48.9 | 29.5 | 16.6 | 74 | 4.3 | 5.3 | 25.5 | 46.5 | 28.5 | 15.4 | 75.6 |
| Schoten | 84 | 2.8 | 4.2 | 24.4 | 51.2 | 23.3 | 16.5 | 62.9 | 2.7 | 4.5 | 25.6 | 52.3 | 23.4 | 16.3 | 64.2 |
| Sinsin | 11 | 3.0 | 1.0 | 10.6 | 56.4 | 16.8 | 14.1 | 83.5 | 2.9 | 1.2 | 13.2 | 61.7 (19000) | 19.6 | 13.8 | 81.3 |
| Ukkel | 4 | 5.0 | 3.2 | 19.7 | 62.6 (20815) | 24.5 | 16.5 | 73.3 | 3.9 | 3.9 | 21.1 | 57.0 (20000) | 21.9 | 16.4 | 70.9 |
| Veurne | 41 | 1.8 | 1.4 | 12.9 | 54.1 | 24.8 | 10.8 | 82.2 | 1.8 | 1.8 | 12.2 | 52.6 | 23.6 | 14.4 | 83.3 |
| Voorhaven | 25 | 3.7 | 21.7 | 39.9 | 42.8 | 33.7 | 16.1 | 72.1 | 2.6 | 19.9 | 35.8 | 41.3 | 30.1 | 14.7 | 73 |
| Zwevegem | 43 | 1.8 | 3.2 | 18.5 | 49.9 | 30 | 11.5 | 78.5 | 1.8 | 4.4 | 19.9 | 46.7 | 26.8 | 15.4 | 78.6 |

#### 4.2.3.4 Leaf wettability

Leaf wettability or hydrophobicity can be determined by measuring the contact angle (CA, °) of water droplets (7 µl) with the leaf surface (Brewer et al. 1991, Fig. 4.2). At each monitoring station, shoots were cut and immediately transported in water-filled tubes to the laboratory, where the leaves were carefully excised from the shoots to avoid wax damage. For the first in-leaf season, one segment was excised from ten randomly selected leaves and mounted onto glass slides using double sided tape, with the abaxial surface facing up. For the second in-leaf season, two segments were excised from each of ten randomly selected leaves. One segment from each leaf was mounted onto glass slides with the abaxial surface facing up, while the other segment was mounted with the adaxial surface facing up. The software ImageJ (Dropsnake analysis) was used to calculate the CAs from digital photographs (Fig. 4.2) of water droplets on the leaf surfaces (Canon EOS 5D digital camera and Sigma macro lens EX DG 105 mm, f/2.8).

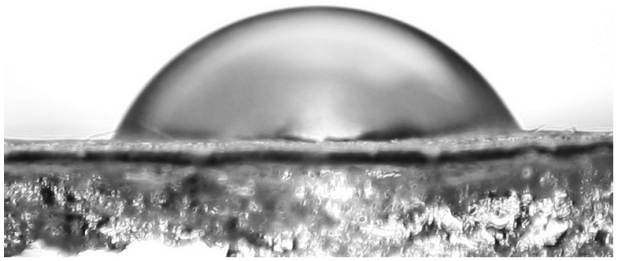

Figure 4.2: Digital photograph of a leaf droplet on the leaf surface of white willow

#### 4.2.3.5 Chlorophyll fluorescence

Chlorophyll fluorescence measurements were made in situ using a portable plant stress fluorometer (Handy PEA, Hansatech Instruments, Norfolk, UK; Fig. 4.3) with a saturating light intensity of 3000 µmol $m^{-2}$ $s^{-1}$. Fully expanded and intact leaves were dark-adapted for 30 min to ensure that the maximum level of fluorescence was reached using clips with a dark room. At each monitoring station, 20 leaves were selected randomly during the first in-leaf season and 15 leaves were selected randomly for the measurements during the second in-leaf season. After dark adaptation, the minimum ($F_o$) and maximum ($F_m$) fluorescence and the performance index (PI) were measured on the adaxial leaf surface. The maximum photochemical

efficiency of photosystem II ($F_v/F_m$) was calculated as ($F_m$ - $F_o$)/$F_m$, with $F_v$ as variable fluorescence.


Figure 4.3: Portable stress fluorometer and clips with a dark room (www.hansatech-instruments.com)

#### 4.2.3.6 Shade, herbivory and vapor pressure deficit

At each monitoring station, digital hemispherical photographs were taken (Nikon D70s with Sigma circular fisheye 8 mm f/4 EX DG). From these photographs, canopy openness (%) was calculated using Gap Light Analyzer software (www.ecostudies.org/gla). The degree of shade was calculated as 100% - canopy openness at the level of monitoring station (Table 4.2).

A measure for herbivory damage was calculated during the first in-leaf season as the ratio of the amount of damaged leaves to the total amount of leaves (%). The herbivory index was determined on the shoot level; a shoot is defined as an individual branch emerging from the original stem cutting.

Meteorological data, i.e., air temperature (T, °C) and relative air humidity

(RH, %) were obtained from weather stations in the vicinity of each air quality monitoring station and operated by the Royal Meteorological Institute (www.kmi.be). Vapor pressure deficit (VPD, Pa) of the air was calculated for each monitoring station for the first and second in-leaf season using Eq. 4.1 (Murray 1967).

$$VPD = \frac{100 - RH}{100} \times 610.7 \times 10^{7.5T/(273.3+T)} \tag{4.1}$$

### 4.2.4 Statistical analysis

Environmental conditions and plant characteristics were compared between the first and second in-leaf season using a Spearman rank correlation test. A paired t-test was used to determine whether the value was higher or lower in the second in-leaf season than in the first in-leaf season.

Because of the hierarchical nature of the data, we used linear mixed models to relate the leaf characteristics to the set of explanatory variables. Several leaves were sampled on the same shoot, multiple shoots occurred on a stem cutting, and multiple stem cuttings were grown per monitoring station; hence, shoot level was nested within stem cutting, which was nested within monitoring station. The determination of the optimal random and fixed effect model was done according to the methodology described in §2.2.3. To analyze SLA, FAA, $R_S$, SD, SPS, CA, $F_v/F_m$ and PI, we used PCA1, VPD, shade, herbivory and their first order interactions with PCA1 as explanatory variables. For the stomatal characteristics, SLA was also used as an explanatory variable; SD can co-vary with SLA (Loranger and Shipley, 2010), which might complicate interpretations of variation in SD. All of the analyses were performed using a 5% significance level and run with the nlme package in R 2.13.1 statistical software (R Development Core Team 2011).

## 4.3 Results

### 4.3.1 Air quality

In general, $O_3$ concentrations were negatively related with $NO_2$ ($r^2 = 0.633$), $PM_{10}$ ($r^2 = 0.345$) and $SO_2$ ($r^2 = 0.009$) concentrations. Further, $NO_2$ concentrations were positively related with $PM_{10}$ ($r^2 = 0.423$) and $SO_2$ ($r^2 = 0.126$) concentrations, and $PM_{10}$ concentrations were also positively related with $SO_2$ concentrations ($r^2 = 0.336$). Consequently, as rural areas are dominated by a higher $O_3$ concentration and urban areas by a higher

$NO_x$ and PM concentration (Fuhrer and Bungener 1999, see §1.1.2), it is difficult to distinguish between less and more polluted areas. The principal component analyses, for obtaining a site specific value for the ambient air quality, gave rise to two principal components axes with an eigenvalue larger than one (Fig. 4.4). The first principal component (PCA1) had a positive loading for the mean NO and $NO_2$ concentration and a negative loading for the mean $O_3$ concentration. The second principal component (PCA2) had a positive loading for the mean $SO_2$ concentration. The mean $PM_{10}$ concentration was evenly distributed over the two main PCA-axes. PCA1 and PCA2 explained approximately 60% and 20%, respectively, of the total variation in the air quality data. Due to the very low $SO_2$ concentration during both in-leaf seasons and the low contribution of PCA2 to the total variability in air quality data, only PCA1 was considered to be suitable as a site-specific value for the air quality at each monitoring station.

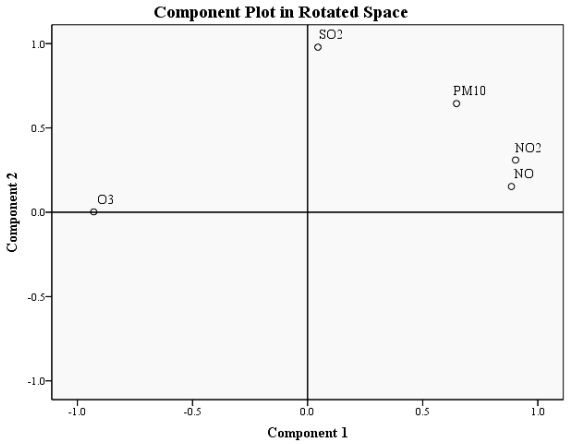


Figure 4.4: Biplot of the mean concentration (μg $m^{-3}$) of $NO_2$, NO, $O_3$, $SO_2$ and $PM_{10}$

The $SO_2$ concentrations never exceeded the hourly and daily limit values for protecting human health (see §1.1.3.1), during the first and second in-leaf season. $PM_{10}$ concentration exceeded the daily limit value most frequently at the 'Voorhaven' monitoring station and $PM_{10}$ values in excess of the limit values were measured 25 and 13 times during the first and second in-leaf season, respectively. For $NO_2$, the yearly limit value for protecting vegetation was exceeded in the monitoring stations 'Borgerhout' and

'Voorhaven'. The $O_3$ limit value for protecting vegetation was also exceeded several times (AOT40, Table 4.2).

### 4.3.2 Comparison of environmental and plant characteristics during first and second in-leaf season

During the first and second in-leaf season, the preliminary analysis of FAA showed that (i) directional asymmetry was not present (one-sample t-test: t = -0.235, p = 0.815 and t = -1.34, p = 0.181, respectively), (ii) antisymmetry was not present (Kolmogorov-Smirnov: $p < 0.001$ and p = 0.01, respectively), (iii) a leptokurtic distribution was present ($\gamma = 8.204 \pm 0.252$; Bonett-Seier: $p < 0.001$ and $\gamma = 3.679 \pm 0.273$, Bonett-Seier: $p < 0.001$, respectively) and (iv) size correction was necessary (r = 0.480, $p < 0.001$, n = 333 and r = 0.455, $p < 0.001$, n = 318, respectively). Therefore, FAA was calculated as |logRA - logLA| for both in-leaf seasons.

A comparison of environmental and leaf characteristics between the first and second in-leaf season is shown in Table 4.3. The mean CA was significantly lower while the SLA values were significantly higher during the second in-leaf season.

Table 4.3: Mean ± standard deviation of several leaf traits [specific leaf area (SLA, $cm^2\ g^{-1}$), stomatal resistance ($R_S$, $s\ m^{-1}$), stomatal density (SD, $mm^{-2}$), stomatal pore surface (SPS, $\mu m^2$), contact angle (CA, °), leaf area fluctuating asymmetry (FAA), maximum photochemical efficiency of photosystem II ($F_v/F_m$) and performance index (PI)], PCA1 and vapor pressure deficit (VPD, Pa) for the first and second in-leaf seasons and the results of the Spearman rank correlation ($r_s$-value) and paired t-test (t-value) (n = 16), a '*' indicates a significant difference at the 0.05 level

| | First season | Second season | $r_s$ | t |
|---|---|---|---|---|
| PCA1 | See Table 4.2 | See Table 4.2 | 0.939* | 0.001 |
| VPD | See Table 4.2 | See Table 4.2 | 0.85* | -0.467 |
| SLA | 110.9 ± 27.1 | 134.7 ± 20.1 | 0.468 | -3.75* |
| SD | 118.3 ± 20.2 | 70.2 ± 14.7 | 0.754* | 2.65* |
| SPS | 109.2 ± 14.7 | 66.8 ± 8.6 | -0.021 | 1.15 |
| $R_S$ | 76.8 ± 13.7 | 89.2 ± 14.2 | 0.521* | -3.261* |
| CA | 56.4 ± 3.8 | 68.6 ± 5.2 | -0.461 | -0.917* |
| PI | 1.9 ± 0.4 | 2.0 ± 0.7 | 0.376 | -6.242 |
| FAA | 0.03 ± 0.01 | 0.03 ± 0.01 | -0.045 | 0.213 |
| $F_v/F_m$ | 0.84 ± 0.009 | 0.82 ± 0.02 | -0.077 | 2.135 |

The degree of shade was highly correlated with SLA during the first and second in-leaf season. SLA increased with increasing degree of shading, especially when there was more than 80% shadow. Therefore, to minimize the confounding effect of shadow, we focused our analysis on the monitoring stations with less than 80% shadow. Removing the monitoring station 'Schoten' was an 'ecological' rather than a 'statistical' choice, since we were not interested in the confounding effect of the degree of shadow on SLA. The PCA was redone on the reduced air quality data and we focused our analysis of SLA and other leaf characteristics on the monitoring stations with less than 80% shade. Tables 4.4-4.5 provide an overview of the results obtained by the mixed model. For each leaf characteristic, the between-site (level 'monitoring station'), inter-tree (level 'stem cutting') and intra-shoot variability (level 'shoot') are provided, as well as an overview of the explanatory variables that are correlated with the relevant response variable.

SLA was related to PCA1 for both in-leaf seasons (Fig. 4.5a-b) and also $R_S$ was significantly related to SLA and VPD during the first in-leaf season. This strong relationship is a reflection of both the strong relationship between $R_S$ and SD ($r = -0.815$, $p < 0.001$, $n = 127$) and the significant relationship of SD with SLA and VPD. During the second in-leaf season, $R_S$ was related to PCA1 due to the strong relationship between SPS and PCA1 (Fig. 4.6c). It must be noted that the relationship between SPS and PCA1 was influenced by the VPD at each monitoring station because a significant interaction effect of PCA1 and VPD on SPS was found. It is difficult to interpret these results due to the continuity of PCA1 and VPD; however, when VPD is larger than 300 Pa, a negative effect of PCA1 on SPS is found. In contrast to SPS, SD was not related to PCA1, but a correlation with VPD and SLA was found (Fig. 4.6a-b). CA was related to PCA1 during the first in-leaf season, but the weak and non-significant correlation between mean CA and PCA1 ($r = 0.001$, $p = 1.000$, $n = 15$) calls into question this result of the mixed model.

Table 4.4: The percent variation at each level (monitoring station, stem cutting, shoot) that is retained in the optimal random model for all response variables during the first in-leaf season; the significant contribution (indicated by the p- and t-values) of the explanatory variables that are retained in the fixed effect model as well as the percentage of between-site variability that is explained by the explanatory variables; the p- and likelihood ($\chi^2$) values for the ANOVA to compare the fixed-effect model with and without all explanatory variables, n/a: not applicable

| | Variation at each level of the random model | | | Explanatory variable | Explained variability |
|---|---|---|---|---|---|
| | Station | Stem cutting | Shoot | | |
| SLA | 80 | 12 | n/a | PCA1 ($p < 0.001$, $t = 2.44$) | n/a |
| FAA | 7 | 93 | n/a | None ($p = 0.829$, $\chi^2 = 2.25$) | n/a |
| $R_S$ | 41 | 11 | 43 | SLA ($p = 0.0267$, $t = 2.525$) | 75 |
| | | | | VPD ($p = 0.0159$, $t = 2.805$) | |
| SD | 41 | 17 | n/a | SLA ($p = 0.0119$, $t = -2.96$) | 92 |
| | | | | VPD ($p < 0.001$, $t = -5.75$) | |
| SPS | 12 | n/a | n/a | None ($p = 0.233$, $\chi^2 = 9.282$) | n/a |
| CA | 8 | n/a | n/a | PCA1 ($p = 0.0273$, $t = -2.23$) | 41 |
| $F_v/F_m$ | 57 | 5 | 23 | Shade ($p = 0.0034$, $t = 3.574$) | 53 |
| PI | 28 | 20 | 23 | None ($p = 0.100$, $\chi^2 = 15.41$) | n/a |

Table 4.5: The percentage of variation at each level (monitoring station, stem cutting, shoot) that is retained in the optimal random model for all response variables during the second in-leaf season; the significant contribution (indicated by the p- and t-values) of the explanatory variables that are retained in the fixed effect model as well as the percentage of inter-site variability; the p- and likelihood ($\chi^2$) value for the ANOVA to compare the fixed effect model with and without all explanatory variables; n/a: not applicable

| | Variation at each level of the random model | | | Explanatory variable | Explained variability |
|---|---|---|---|---|---|
| | Station | Stem cutting | Shoot | | |
| SLA | 44 | 4 | 18 | PCA1 (p = 0.003, t = 3.65) | n/a |
| FAA | 6 | 5 | 15 | None (p = 0.538, $\chi^2$ = 7.96) | n/a |
| $R_S$ | 17 | 12 | n/a | PCA1 (p < 0.001, t = 4.96) | 90 |
| SD | 29 | 26 | n/a | SLA (p = 0.002, t = -3.99) | > 90 |
| | | | | VPD (p = 0.0066, t = -3.27) | |
| SPS | 12 | n/a | n/a | PCA1:VPD (p = 0.0031, t = -3.687) | 90 |
| $CA_{ad}$ | < 0.1 | n/a | n/a | Shade (p = 0.048, t = -2.17) | 50 |
| $CA_{ab}$ | 8 | 34 | n/a | None (p = 0.295, $\chi^2$ = 11.86) | n/a |
| $F_v/F_m$ | 42.5 | 17 | n/a | Shade (p = 0.0004, t = 3.52) | 79.6 |
| PI | 38 | n/a | n/a | Shade (p = 0.0162, $\chi^2$ = 2.796) | 59.7 |

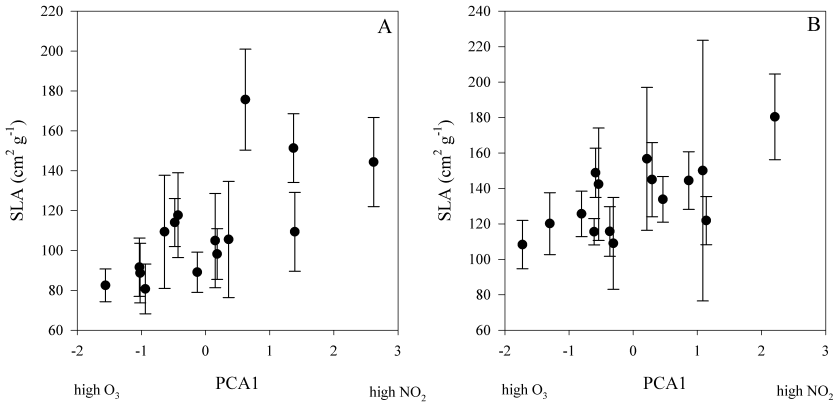


Figure 4.5: Mean and standard deviation of specific leaf area (SLA) for white willow at the monitoring stations (< 80% shade) as a function of PCA1 ($NO_2/O_3$) for the (a) first and (b) second in-leaf seasons

## 4.4 Discussion

The set-up of the experiment enabled us to block out, reduce or, at least, evaluate and take into account the effect of confounding genetic and environmental stressors on FAA and SLA. Because stem cuttings were used, the genetic component had a marginal influence on the observed FAA and SLA values. The efficient semi-automatic water supply system prevented drought stress, and differences in soil characteristics were omitted by the use of uniform potting soil. Although snail herbivory was excluded with copper tape, leaves were often damaged by caterpillars and/or aphids.

### 4.4.1 Specific leaf area

The large influence of shade on SLA, during both in-leaf seasons, reflects the importance of taking light levels into account in biomonitoring studies. Under shaded conditions, carbon uptake per unit leaf biomass is lower than under full light conditions (Van Hees and Clerkx 2003). To maintain a positive C balance, biomass partitioning, physiological adjustments, and/or morphological and anatomical adjustments can be developed (Van Hees and Clerkx 2003). Morphological and anatomical adjustments are well known adaptations to long-term shade: shade leaves are thinner than

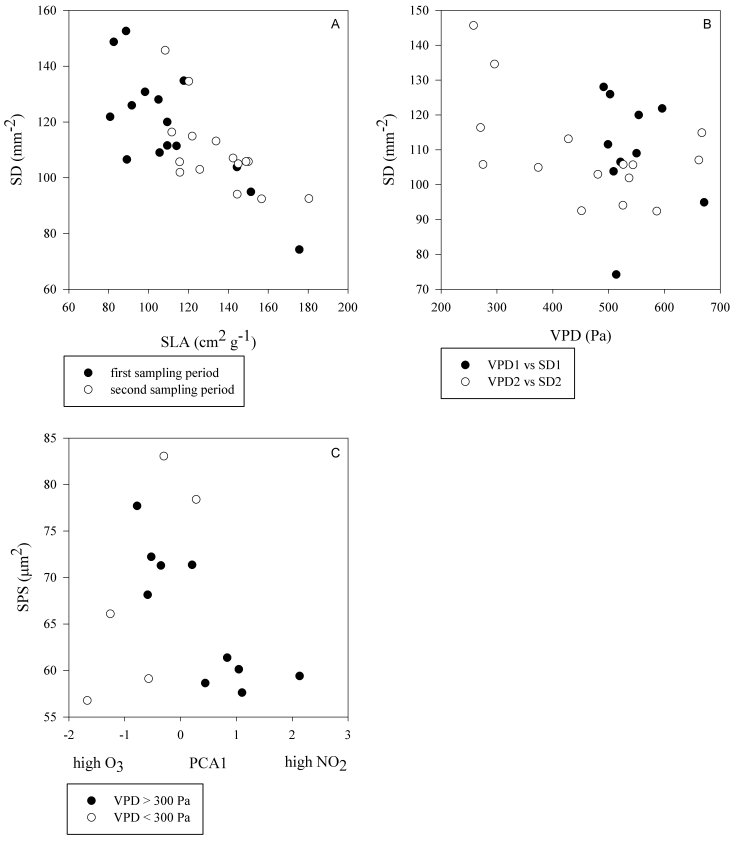


Figure 4.6: Mean stomatal density (SD) for white willow at the monitoring stations (< 80% shade) as a function of (a) specif leaf area (SLA) and (b) vapor pressure deficit (VPD) for both the first and second in-leaf seasons and means of stomatal pore surface (SPS) for white willow as a function of (c) PCA1 for the second in-leaf season

sun leaves, due to the reduction of the amount of palisade cell layers, and have a higher leaf area for absorption of photosynthetic active radiation, causing a higher SLA. The cross-sections of the leaves (Fig. 4.7) illustrate a reduction in the palisade parenchyma thickness rather than a reduction of the spongy parenchyma as a response to shade.
SLA was also significantly related to PCA1 during the first and second in-leaf season. SLA was highest at 'Dourbes', which had the highest atmospheric $O_3$ and lowest atmospheric $NO_2$ concentration, while SLA was lowest at 'Voorhaven', which had the highest atmospheric $NO_2$ and the

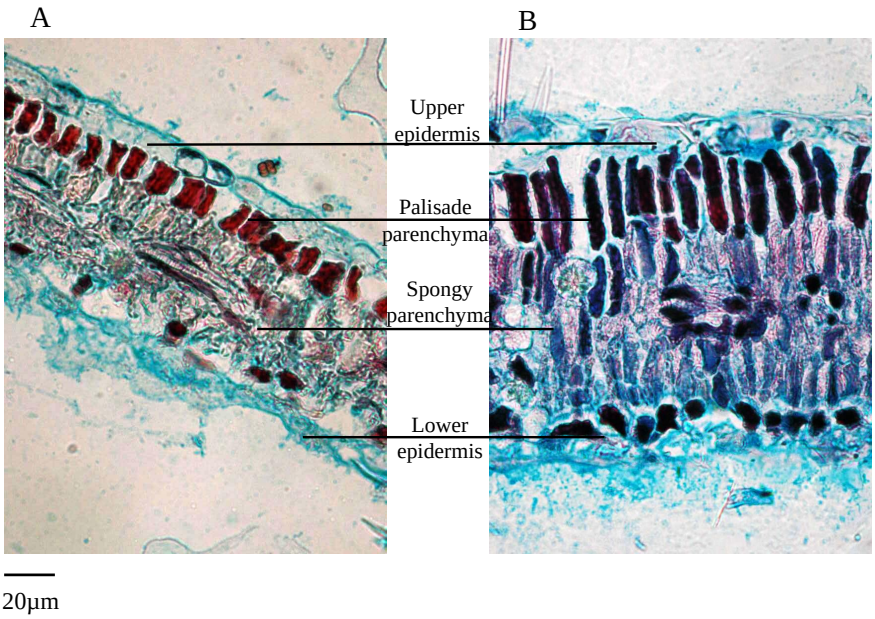


Figure 4.7: Morphology of cross-sectional images through the leaf-blade region of fully expanded white willow leaves in (a) shaded and (b) open habitats using light microscopy

lowest atmospheric $O_3$ concentration (Table 4.2). The effect of air pollution on SLA is species-dependent and related to the protective or adaptive mechanism of plants. For example, Wen et al. (2004) showed that SLA of *Machilus chinensis* increased and SLA of *Ilex rotunda* and *Ficus microcarpa* decreased due to air pollution. Also, Poorter et al. (2009) stated that, in general, a high $O_3$ concentration increased SLA of monocots and decreased SLA of dicots. Since research about the effect of air pollution on SLA of *Salix* sp. is to our knowledge never performed, the protective or adaptive mechanism of white willow is not known. On the one hand, it is possible that white willow decreased SLA for minimizing the uptake of air pollutants (Wen et al. 2004) by decreasing leaf area, decreasing density and/or thickness (Tiwari et al. 2006), and/or increasing leaf starch concentration (Schmitt et al. 1999), due to a high atmospheric $O_3$ concentration. On the other hand, it is possible that white willow increased SLA, due to a compensatory growth, that occurred to reduce the inhibition of photosynthesis (Canas et al. 1997), caused by a high $NO_x$ concentration. The

latter hypothesis is supported by previous results in Chapter 3: $R_S$ of white willow decreased, as a consequence of the higher atmospheric $NO_x$ concentration in urban areas, compared to rural areas. In addition, Jäger et al. (1992) found that, at comparable atmospheric $NO_x$ (and $O_3$) concentration with our study, protein content was enhanced due to $NO_x$ foliar uptake, and, therefore, a fertilization effect of $NO_x$ on SLA of white willow cannot be ignored as a possible hypothesis. Also, Knops and Reinhart (1999) stated that N fertilization can, to some point, positively influence growth and cause an increase in SLA.

In our study, it is possible that the white willow leaves changed their SLA by changing leaf thickness, rather than leaf density, as a response to air pollution. The thickness of the upper and lower epidermis is comparable between Dourbes' (high atmospheric $O_3$ and low $NO_2$ concentration) and 'Voorhaven' (high atmospheric $NO_2$ and low $O_3$ concentration) (± 20 μm; Fig. 4.8); however, the parenchyma of the leaves at 'Dourbes' consists of larger cells (Fig. 4.8a), compared to 'Voorhaven' (Fig. refcrossb). The total parenchyma thickness at 'Dourbes' was 183 μm, while the thickness at 'Voorhaven' was 164 μm. A reduction of parenchyma thickness was also found by Rashidi et al. (2012), who recorded a flattening of the spongy parenchyma cells in polluted areas. Based on Fig. 4.8, we can suggest that leaf density is similar between 'Dourbes' and 'Voorhaven', but leaf density measurements must be performed to confirm this statement. The dark contents, especially in the mesophyll cells, are probably phenolic compounds. This observation was also made by Gostin and Ivanescu (2008). Zobel et al. (1996) showed that biotic and abiotic stressors can induce an increase in phenolic compounds; this finding was also confirmed by our study (Chapter 5).

Besides the individual effect of $NO_x$ and $O_3$, also possible synergistic or antagonistic effects need to be considered. For example, Tiwari et al. (2006) stated that, although the individual atmospheric $SO_2$, $NO_x$ and $O_3$ concentrations are below the limit value for plant injury, the combined effect of the pollutants seems to act synergistically and decrease plant growth. Muzika et al. (2004) found a relation between atmospheric $O_3$, $NO_2$ and $SO_2$ concentrations and growth, but a more complete relationship appeared when the air pollution variables were combined, showing a multiplicative effect. Also, an antagonistic effect of $NO_2$ and $O_3$ was found by Jäger et al. (1992), who showed that $O_3$ exposure counteracted the positive effect of relatively low $NO_2$ levels.

Combining our observations of PCA1 - which is negatively correlated with atmospheric $O_3$ concentration and positively correlated with atmospheric

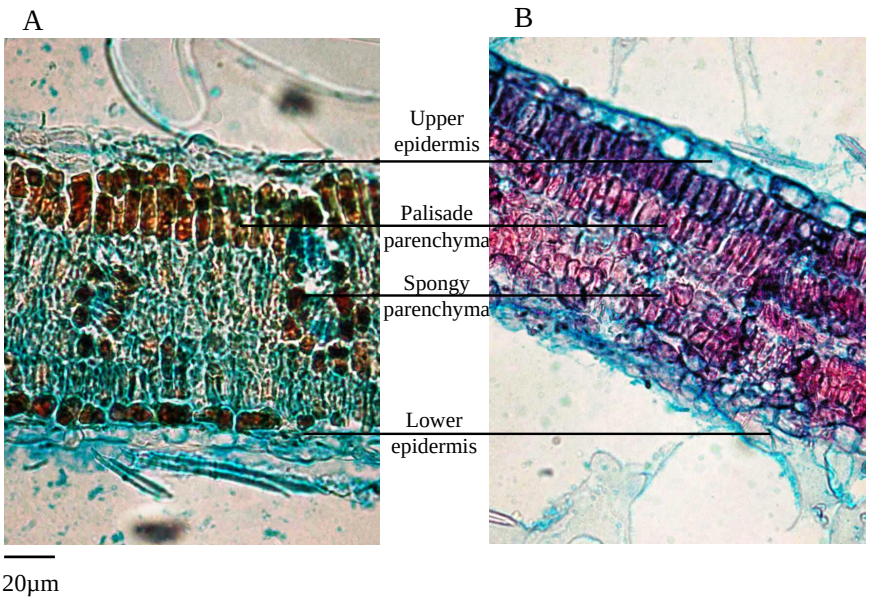


Figure 4.8: Morphology of cross-sections through the leaf-blade region of fully expanded leaves of white willow at (a) 'Dourbes' (high $O_3$ and low $NO_2$ concentration) and (b) 'Voorhaven' (high $NO_2$ and low $O_3$ concentration) using light microscopy

$NO_2$ concentration - and the contradictory results in the literature, we can only conclude that the combination of the ambient $NO_x$ and $O_3$ concentration induced changes in SLA of white willow. However, from our dataset it is not possible to determine which of the pollutants ($NO_x$ or $O_3$) is most important in controlling the response of white willow or whether the change in SLA is the result of a synergistic or antagonistic interaction between $NO_x$ and $O_3$. For that purpose we would need to perform controlled fumigation experiments with low ambient pollutant concentrations.

We also found that SLA was generally higher during the first in-leaf season compared to the second in-leaf season. Although mean VPD and PCA1 were comparable between the first and second in-leaf season, this does not imply the absence of a possible effect by different daily and/or monthly T, RH and/or PCA1. Moreover, nutritional depletion and/or harvest of willows during the first in-leaf season could have posed some additional stress to the plants during the second in-leaf season, which could be the cause

of the difference in SLA between the first and second in-leaf season. The production of leaves with a higher SLA after defoliation has been reported by several studies (e.g., Cuni Sanchez et al. 2010). The yellowing of the leaves and the dwarf growth due to unfavorable growth conditions in the third in-leaf season strengthen this hypothesis.

### 4.4.2 Fluctuating asymmetry

The small between-site variability of FAA could not be explained by herbivory, T, RH, shade and/or PCA1, and, therefore, we can conclude that FAA is no good biological indicator for assessing low concentrations of air pollutants. Reasons may be (i) a too small sample size, (ii) too low concentrations of air pollutants, and/or (iii) insusceptibility of white willow to air pollutants, in terms of FAA. Firstly, although Hodar (2002), Kozlov et al. (2002) and Hagen et al. (2008) used a small sample size (n = 10, 15, 10 respectively), Mogie and Cousins (2001) stated that large sample sizes (several hundred leaves) may be required for reliable estimates of FAA. A retrospective power analysis showed that the statistical power was only 14% and that a sample size of 84 was necessary to obtain a power of 80%. Also, Van Dongen (1999) showed that, with two repeats and a sample size of 20, statistical power hardly exceeded the nominal level. Therefore, the small sample size probably lacked the statistical power to detect a relationship between small differences in FAA and air pollution. However, a large sample size, makes the use of FAA not time- and cost-effective, and, therefore, not useful for the biomonitoring purposes we envisioned. Secondly, regarding low air pollutant concentrations, according to Parsons (1992) severe stress is necessary to increase FA under field conditions, leading to the inability of FAA to detect the low atmospheric pollutant concentrations at the monitoring stations. Moreover, Raz et al. (2011) stated that FA seems to be a less sensitive indicator of stress than physiological and morphological plant characteristics, since they evolve to minimize or buffer even minor stress. Therefore, it is possible that the ambient air pollution caused adaptive modifications (e.g., SLA) to buffer stress, without exceeding the homeostatic abilities of white willow. Thirdly, it is possible that FAA of white willow is insensitive to air pollution. In literature, a few studies investigated the effect of stress factors on FA of willow species, but no relationship was found. For example, Hochwender and Fritz (1999) found no influence of water stress, pathogen attack and competition on leaf FA of *Salix* hybrids. Also, Dimitriou et al. (2006) found no relationship between leaf FA and landfill leachate of several willow clones and Zvereva and Kozlov (2001) stated that leaf FA of *Salix borealis* was no good indicator of air pollution

load.

### 4.4.3 Stomatal characteristics

During the first and second in-leaf season, SD was lower at monitoring stations with a higher VPD (Fig. 4.6a-b), which led to an increase in $R_S$ and thus a lower transpirational water loss due to a dry atmosphere. Because VPD is positively correlated with mean T and negatively with mean RH, SD is lower at the monitoring stations with a higher T and a lower RH. Beerling and Chaloner (1993) and Luomala et al. (2005) also found a negative correlation between the SD and T under which the leaves were formed. PCA1 was also correlated with the SD of white willow (data not shown) during the first and second in-leaf season, but this so-called adaptation of SD to air pollution was attributed to cell expansion rather than stomatal differentiation, because SD negatively co-varied with SLA. If SLA was not taken into account, an incorrect conclusion would have been drawn about the effect of air pollution on SD. Therefore, stomatal index measurements (i.e., the ratio of the number of stomata in a given area divided by the total number of stomata and epidermal cells in that area) should be preferred over measurements of SD, because they have the advantage of taking this co-variation into account. Under normal conditions, the stomatal index is related to the percentage of epidermal cells that become stomata, and this percentage can vary in response to a number of environmental factors (Crispim et al. 2012). Crispim et al. (2012) demonstrated increases in the number of epidermal cells, resulting in decreasing stomatal indexes with higher traffic pollution.

The variation in SPS could not be explained by any environmental factor during the first in-leaf season, whereas PCA1 influenced SPS during the second in-leaf season (Fig. 4.6c). This finding indicates that the accumulated effect of air pollution on SPS outweighs the effect of VPD on SD, causing an increase in $R_S$. Increasing $R_S$ as a consequence of air pollution can be seen as a method of minimizing atmospheric pollutant uptake while optimizing $CO_2$ uptake and reducing water loss due to transpiration. Because our white willow stems increased their $R_S$ at monitoring stations with high $NO_2$ concentrations, we can suggest that $NO_2$ is the main cause of air pollution stress and is, therefore, primarily responsible for the changes in SPS and SLA as well. It must be noted that the negative influence of PCA1 on SPS was only observed when VPD was larger than 300 Pa. We can assume that in drier atmospheric conditions, plants impose stronger stomatal control; as a result, additional environmental stresses likely have a larger impact on SPS. However, the boundary of 300 Pa cannot be explained biologically.

### 4.4.4 Leaf wettability

All higher plants develop a waxy layer in or on the leaf cuticle as a barrier to water loss and/or organic and inorganic compounds. The waxy layer also acts as a reflective coating to reduce leaf surface temperature and as a physical barrier to insect attack or to penetration by fungal hyphae (Cape and Percy 1993). The degree of hydrophobicity or wettability of the epicuticular wax layer is determined by the chemical composition, the physical structure and the quantity of the epicuticular waxes. The abaxial leaf surface of white willow had a lower wettability than the adaxial leaf side, which has also been demonstrated for several other temperate tree species by Kardel et al. (2012). The adaxial leaf surface is directly exposed to a combination of environmental factors, which cause abrasion, erosion or changes in the chemical composition of the wax layer (Kardel et al. 2012). Several studies also showed that air pollution can damage the epicuticular wax layer (Turunen and Huttunen 1990, Kupcinskiene 2001, Schreuder et al. 2001). It is difficult to assess this damage under field conditions, since natural fluctuations in growth, aging, wax re-crystallization, environmental factors (including temperature, relative humidity, shade) and high inter-tree variation can confound the response of leaf wettability to air pollution (Krupa 2003). In our study, the absence of a relationship between the leaf wettability of white willow and the ambient air quality of the first and second in-leaf season likely resulted from a combination of the high inter-tree variability and degree of shade. White willow leaves developed in open habitats were less wettable than leaves formed in shade habitats, which was also found by Cape and Percy (1993) and Pandey and Nagar (2002), indicating the importance of the microclimate in which a plant grows (Koch et al. 2006).

### 4.4.5 Chlorophyll fluorescence

Solar energy absorbed by chlorophyll is dissipated by photochemical or non-photochemical processes. Photochemical processes use the energy for photosynthesis, while non-photochemical processes re-emit the energy in the form of heat and chlorophyll fluorescence. Energy used for photochemical and non-photochemical processes is in equilibrium; as a consequence, when stress reduces photosynthetic performance, energy dissipation via chlorophyll fluorescence increases (Papageorgiou and Govindjee 2004). In our study, $F_v/F_m$ and PI were primarily influenced by the degree of shade under which the leaves were formed, during both in-leaf seasons. Both the $F_v/F_m$ and PI of white willow were higher in shaded habitats compared to open habitats, which can be explained in two ways. Firstly, the

increasing $F_v/F_m$ with decreasing light availability suggests that quantum yield increases in shade-grown plants. This increase would allow more efficient energy transfer from light-harvesting chlorophyll to photosystem (PS) II instead of PS I (Demmig and Bjorkman 1987, Groninger et al. 1996, Eranen and Kozlov 2006). Secondly, it has been demonstrated that a decline in $F_v/F_m$ and PI can indicate the presence of light stress in trees growing in open habitats, resulting in photodamage (Valladares et al. 2002). However, the latter hypothesis seems unlikely for our study because white willow is a pioneer species and is suited to grow in open habitats.

## 4.5 Conclusions

The absence of a clear relationship of FAA, CA and $F_v/F_m$ with ambient air quality indicates that these leaf traits of white willow are unsuitable as monitoring tools, even after exposure to the ambient air pollution for two consecutive years. Both CA and $F_v/F_m$ were affected by the amount of shade, even when the monitoring station with the highest amount of shade (> 80%) was not taken into account. In contrast, SLA and $R_S$ were influenced by the ambient air pollution, indicating that several performance parameters of the same species can respond differently to the same treatment. However, if SLA is used as a biological monitoring tool, the effect of shade must be taken into account by choosing sample sites with similar degrees of shade and by sampling unshaded leaves. If stomatal traits are used as biological monitoring tools, white willow requires a long-term (> 1 yr) exposure period to ambient air pollution. During the first in-leaf season, $R_S$ was mainly influenced by meteorological conditions at each monitoring station, while during the second in-leaf season, ambient air pollution ($NO_2$) affected SPS, and, as a result, also affected $R_S$.

In conclusion, using SLA and/or $R_S$ of white willow has the advantage of being non-destructive, and, therefore, avoids potential stress caused by harvesting. In addition, not harvesting the willows might make it possible to monitor the willows for more than two years, which may lead to a more pronounced effect of the accumulated air pollutants on SLA and/or $R_S$.

# 5

# The influence of ambient air quality on foliar antioxidant system and stable isotopes of white willow

*After: Wuytack, T., AbdElgawad, H., Staelens, J., Asard, H., Boeckx, P., Verheyen, K., Samson, R. The response of the foliar anti-oxidant system and stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) of white willow to low-level air pollution. Submitted in Plant Physiology and Biochemistry*

In this study we aimed to determine and elucidate the effect of ambient air pollution on the foliar antioxidant system and stable carbon ($\delta^{13}$C) and nitrogen isotopes ($\delta^{15}$N) of white willow. We grew white willow in homogeneous potting soil in the near vicinity of sixteen air quality monitoring stations in Belgium where atmospheric $NO_2$, $O_3$, $SO_2$ and PM concentrations were continuously measured. The trees were exposed to ambient air during six months (April - September 2011), and, thereafter, the degree of lipid peroxidation and foliar content of antioxidant molecules, antioxidant enzymes and foliar $\delta^{13}$C and $\delta^{15}$N were measured. We found that lipid peroxidation was caused by oxidative stress, arising from high ambient $NO_2$ concentrations, as shown by an increased amount of mal-

ondialdehyde. The oxidative stress activated the antioxidant system by increasing the amount of polyphenols, while no increase of key enzymes scavenging reactive oxygen species was observed. The influence of atmospheric $NO_2$ was also indicated by a decrease of $\delta^{13}C$ with increasing atmospheric $NO_2$ concentrations, probably reflecting a decreased net photosynthesis and/or a concomitant decrease of $^{13}CO_2$ in the atmosphere. Shade also influenced foliar $\delta^{13}C$ and the content of leaf ascorbate and glutathione.

## 5.1 Introduction

Air pollutants may generate oxidative stress in plants (Furlan et al. 2010), which in turn alters the intracellular redox environment (Galant et al. 2011) and generates excessive amounts of reactive oxygen species (ROS) (Mittler 2002). ROS are not only comprised of free superoxide ($O_2{}^{-1}$) and hydroxyl radicals but also of molecules such as hydrogen peroxide ($H_2O_2$), singlet oxygen and $O_3$ derived from photorespiration, the photosynthetic apparatus and mitochondrial respiration (Mittler 2002, Blokhina et al. 2003). This enhanced ROS production can pose a threat to cells, giving rise to membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damage (Mittler 2002, Miller et al. 2010). Lipid peroxidation leads to the production of malondialdehyde (MDA), which is seen as an indicator for a variety of abiotic and biotic stresses (Munné-Bosch and Alegre 2003, Apel and Hirt 2004). To protect cells under stressful conditions, plant tissues contain enzymes for scavenging ROS (e.g., superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX)) and low-molecular mass antioxidants (e.g., reduced ascorbate (ASC) and glutathione (GSH), tocopherols, flavonoids (FLA) and phenols (POLY)) (Blokhina et al. 2003, Miyake 2010). Scavenging of $O_2{}^{-1}$ radicals is achieved by SOD, while $H_2O_2$ is scavenged by CAT, APX, various other POX and POLY compounds (Blokhina et al. 2003). Leaf POLY can also decrease the fluidity of the membranes to hinder diffusion of free radicals (Arora et al. 2000). For example, Tiwari and Agrawal (2011) found an increase of leaf ASC and POLY after $O_3$ exposure, which suggested the triggering of a defense mechanism to high atmospheric $O_3$ concentrations.

Air pollutants may also generate changes in the foliar stable isotope values (e.g., Balasooriya et al. 2009), making isotopes a powerful tool for advancing our understanding of relationships between plants and the environment (Dawson et al. 2002). Two naturally occurring stable isotopes of carbon

(C), $^{13}C$ and $^{12}C$, and nitrogen (N), $^{14}N$ and $^{15}N$, are present in the atmosphere. Most of the atmospheric C is $^{12}C$ (98.9‰), with 1.1‰ being $^{13}C$ (Farquhar et al. 1989) and most of the nitrogen is $^{14}N$ (e.g., 99.63‰ in atmospheric $N_2$) (Dawson et al. 2002). Anthropogenic activities can affect atmospheric $^{13}C$ and $^{15}N$ (Ammann et al. 1999, Balasooriya et al. 2009), and hence affect the foliar $\delta^{13}C$ and $\delta^{15}N$ (Robinson 2001, Kwak et al. 2009). In addition, air pollutants can alter $\delta^{13}C$ by influencing C discrimination (Rennenberg and Gessler 1999) during stomatal conductance and/or carboxylation (Dawson et al. 2002). For example, Balasooriya et al. (2009) found a decreased $\delta^{13}C$ with increasing urbanization, while Battiplagia et al. (2010) found an increase of $\delta^{13}C$ with increasing concentrations of $SO_2$, $NO_x$ and $CO_2$. A positive delta value indicates that the leaf contains more of the heavy isotope ($^{13}C$ and $^{15}N$) than the standard, whereas a negative delta value indicates that the leaf contains less of the heavy isotope than the standard (Dawson et al. 2002).

Our knowledge about these biochemical adaptations of plants, caused by the exposure to air pollution, is mostly based on experiments where plants have been exposed to high concentrations of a single air pollutant during short periods, provoking acute damage under experimental conditions. Less information is gathered about the response of biochemical plant characteristics on longer-term exposure to multiple ambient air pollution sources under field conditions. Therefore, the main objective of this study was to investigate the response of biochemical leaf characteristics of white willow to the ambient air quality and to evaluate the potential of these leaf characteristics as effective parameters for biomonitoring the ambient air quality. To our knowledge, this is the first report of the antioxidant system and stable C and N isotope response of white willow to ambient air quality. We hypothesized that (i) oxidative stress, due to air pollution, caused an increased activity of enzymes and/or an increased content of antioxidants and (ii) $\delta^{13}C$ and $\delta^{15}N$ values were affected by the level of ambient air pollution.

## 5.2 Materials and methods

### 5.2.1 Study area and experimental design

White willow cuttings (n = 12) were grown in the near vicinity of air quality monitoring stations in Belgium (see §1.1.3.2, Fig. 1.3, Table 4.1). Cuttings were planted in 3.5 $dm^3$ pots with homogeneous potting soil as described in §4.2.2. During the sampling period (April - September 2011), the plants were well watered by using a semi-automatic water supply system (see

§4.2.2) and copper tape was used to avoid snail herbivory.

### 5.2.2 Data acquisition

#### 5.2.2.1 Air pollutant concentration and meteorological data

The atmospheric $NO_2$, $O_3$, $SO_2$ and $PM_{10}$ concentrations ($\mu g\ m^{-3}$) were obtained from the air quality monitoring stations (Table 5.1) and data about the air temperature (T, °C) and relative air humidity (RH, %) were obtained from weather stations in the vicinity of each monitoring station (Table 5.1). For the degree of shade, we refer to Table 4.2.

A principal component analysis was performed to reduce the amount of air quality data (see §4.2.3.1) and one principal component axis (PCA1) was retained as a site specific value for the air quality at each monitoring station (Table 5.1). Axis PCA1 had a positive loading for the mean NO and $NO_2$ concentrations in the period April-September 2011 and a negative loading for the mean $O_3$ concentration and explained 60% of the total variability of the air quality data.

Table 5.1: Mean concentration of atmospheric $SO_2$, NO, $NO_2$, $O_3$ and $PM_10$ ($\mu g\ m^{-3}$), mean air temperature (T, °C) and mean relative air humidity (RH, %) during the sampling period (April to September 2011); PCA1 is a score along the first principal component axes for air pollution.

| Station | $SO_2$ | NO | $NO_2$ | $O_3$ | $PM_{10}$ | PCA1 | T | RH |
|---|---|---|---|---|---|---|---|---|
| Aarschot | 1.71 | 2.66 | 15.95 | 58.22 | 20.45 | -0.62 | 16.25 | 71.80 |
| Berendrecht | 3.02 | 7.21 | 26.01 | 50.27 | 27.99 | 0.50 | 16.35 | 69.00 |
| Borgerhout | 2.90 | 8.98 | 38.36 | 45.97 | 24.97 | 1.12 | 16.6 | 64.00 |
| Charleroi | 1.02 | 4.47 | 22.79 | 49.45 | 22.69 | 0.23 | 16.17 | 72.12 |
| Corroy | 1.56 | 2.54 | 16.68 | 47.00 | 21.62 | -0.02 | 15.42 | 81.67 |
| Dourbes | 0.46 | 0.14 | 4.52 | 68.04 | 14.21 | -1.73 | 14.95 | 83.17 |
| Engis | 5.74 | 2.98 | 18.18 | 57.47 | 31.42 | -0.4 | 16.65 | 68.66 |
| Hasselt | 1.65 | 5.56 | 21.55 | 55.45 | 17.38 | -0.24 | 15.48 | 66.67 |
| Mendonk | 2.69 | 4.14 | 24.06 | 50.05 | 34.1 | 0.48 | 16.38 | 74.33 |
| Mons | 0.69 | 7.5 | 24.93 | 45.57 | 18.39 | 0.55 | 15.92 | 72.00 |
| Schoten | 6.43 | 4.19 | 24.17 | 53.83 | 22.1 | -0.29 | 16.24 | 65.14 |
| Sinsin | 1.67 | 1.12 | 8.17 | 62.09 | 16.39 | -1.28 | 14.83 | 82.25 |
| Ukkel | 6.24 | 3.43 | 20.68 | 58.27 | 21.09 | -0.68 | 15.42 | 81.67 |
| Veurne | 1.50 | 1.91 | 11.57 | 55.70 | 24.99 | -0.54 | 15.17 | 84.89 |
| Voorhaven | 2.56 | 25.06 | 37.84 | 39.74 | 34.06 | 2.60 | 15.63 | 71.50 |
| Zwevegem | 1.46 | 3.73 | 20.16 | 46.97 | 26.92 | 0.33 | 15.88 | 79.85 |

#### 5.2.2.2 Lipid peroxidation and antioxidant system

In September 2011, leaves were randomly collected at each monitoring station by excision with a clean scissor from different sides of each willow (n = 12). The collected leaves were placed in labeled plastic bags and stored in liquid nitrogen ($N_2$). After immediate transport to the laboratory, samples were stored at -80°C until extraction. To prepare the extract, leaves were ground in liquid $N_2$ and 0.100 g of the leaf powder was weighed into a reaction tube previously cooled in liquid $N_2$. For the determination of the total antioxidant capacity (FRAP), 0.200 g plant material (FW) was weighed.

Leaf MDA (nmol $g^{-1}$ FW), formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of lipid peroxidation (Mittler 2002). MDA content was estimated by following the protocol of Gautier et al. (2010) and Murshed et al. (2008).

The determination of FRAP is based on the reduction of ferric tripyridyltriazine (TPTZ) complex to the ferrous TPTZ at low pH. This ferrous TPTZ complex has an intensive blue color that can be monitored (Benzie and Strain 1996). The plant tissues were ground in liquid $N_2$ and the antioxidants were extracted in 2ml of ice cold 80% ethanol. 180 µl of FRAP reagent (0.3 M acetate buffer (pH 3.6), 0.01 mM TPTZ in 0.04 mM HCl and 0.02 M $FeCl_3.6\ H_2O$) mixed with 20 µl extract and measured at 600 nm using a microplate reader. Trolox (0 to 650 µM) was used as standard and total non-enzymatic antioxidant activity was quantified as µmol trolox $g^{-1}$ FW.

Leaf POLY and FLA were extracted in 80% ethanol (v/v). The POLY content of each extract was expressed as mg gallic acid equivalents per gram FW and determined according to the micro-plate method of Zhang et al. (2006). The FLA content was expressed as quercetin equivalents per gram FW and measured at 415 nm according to the method of Chang et al. (2002).

Leaf ascorbate and glutathione were extracted in 1 ml of 6% (w/v) metaphosphoric acid. Oxidized forms were reduced by using a mixture of 200 mM dithiothreitoland 400 mM Tris solution, leading to the formation of ASC and GSH. The determination of ASC (µmol $g^{-1}$ FW) and GSH (µmol $g^{-1}$ FW) was carried out by reverse phase liquid chromatography (HPLC; RP type C-18 column, LiChroSpher, Alltech; isocratic pump, 0.8 mL $min^{-1}$, LCADVP, Shimadzu, Columbia). The HPLC was coupled to an electrochemical detection system (reference potential 1,000 mV) and UV detection system (SPD-M10Avp, Diode Array detector). Chromatogram analy-

sis was performed with Class VP software (ClassVP 5.0, Shimadzu).

Antioxidant enzymes were extracted in 1 ml of cold extraction mixture of 50 mM MES/KOH buffer (pH 6.0) containing 40 mM KCl, 2 mM $CaCl_2$ and 1 mM L-ascorbic acid, according to the method of Torres and Andrews (2006). 200 mg of liquid $N_2$ frozen plant tissue was homogenized in a MagNA Lyser (Roche, Vilvoorde, Belgium, 1 min, 7000 rpm). The homogenate was centrifuged at 14 000 g for 10 min at 4°C, from which the supernatant was used as a source for both soluble protein and crude enzymes. Soluble protein content was determined according to the method of Lowry et al. (1951).

The SOD activity per mg protein was determined according to a modified method of Dhindsa et al. (1981) and measured by using the microplate method with a 0.2 ml reaction mixture (50 mM potassium phosphate (pH 7.8) buffer, 13 mM methionine, 75 M nitro blue tetrazolium, 0.1 mM EDTA, 20 µL supernatant and 2 µM riboflavin). The SOD activity was determined by measuring NBT reduction at 560 nm with a spectrophotometer. The activity was quantified by using a standard curve using known amounts of purified SOD enzyme under identical conditions against the % of NBT reduction (we dilute our samples (< 50%) to set in the linear part of the standard curve).

The POX activity, expressed as pyrrogalloline formed $min^{-1}$ $mg^{-1}$ protein, was estimated according to the method of Kumar and Khan (1982) and was determined in a reaction mixture (0.05 M phosphate buffer (pH 6.8), 0.01 M pyrogallol, 10 µl supernatant and 0.01 M $H_2O_2$) by measuring the decrease of the reaction rate at $A_{430}$. The APX activity, expressed as ascorbate formed $min^{-1}$ $mg^{-1}$ protein, was determined according to the method of Murshed et al. (2008) in a reaction mixture of 190 µL (50 mM potassium phosphate buffer (pH 7.0), 0.25 mM AsA, and 5 mM $H_2O_2$) and 10 µL supernatant, by measuring the decrease in the reaction rate at $A_{290}$ then calculated from the 2.8 $Mm^{-1}cm^{-1}$ extinction coefficient.

#### 5.2.2.3 Stable N and C isotopes

In September 2011, leaves were randomly selected from each willow at each monitoring station, immediately stored in liquid $N_2$ and transported to the laboratory (n = 12). Stable isotopes were analyzed for a composite sample of dried leaves (24 h at 80°C) from all willows at each monitoring station. Composite samples were ground using a centrifugal mill (MM200, Retsch, Germany). Subsamples were weighed in tin cups and analyzed in

duplicate for total C (%), N (%), $\delta^{13}$C (‰) and $\delta^{15}$N (‰) using an elemental analyzer (EA) (ANCA-SL, SerCon, UK) coupled to an Isotope Ratio Mass Spectrometer (IRMS) (20-20, SerCon, UK). In addition, soil samples were dried, ground and analyzed (C, N, $\delta^{13}$C, $\delta^{15}$N) in the same way as the leaves.

### 5.2.3 Statistical analysis

Because of the hierarchical nature of the data, we used linear mixed models to relate the leaf characteristics to the set of explanatory variables as described in §2.2.3. A linear model was preferred in case of a lower AIC value compared to the mixed model.

## 5.3 Results

### 5.3.1 Lipid peroxidation and anti-oxidant system

The mean foliar content of ASC, GSH, MDA, FLA, FRAP, POLY, as well as the mean activity of POX, SOD, APX are given in Table 5.2. The correlations between these biochemical leaf characteristics are given in Table 5.3.

Table 5.2: Mean ± standard deviation (n = 16) of the biochemical leaf characteristics

| Characteristic | Mean ± stdev |
|---|---|
| reduced ascorbate (μmol $g^{-1}$ FW) | 3.21 ± 0.07 |
| reduced glutathione (μmol $g^{-1}$ FW) | 0.068 ± 0.034 |
| malondialdehyde (nmol $g^{-1}$ FW) | 16.8 ± 6.9 |
| flavonoid (mg quercetin $g^{-1}$ FW) | 2.5 ± 1.3 |
| total antioxidant capacity (μmol trolox $g^{-1}$ FW) | 39.9 ± 24.1 |
| polyphenols (mg gallic acid $g^{-1}$ FW) | 16.2 ± 2.3 |
| peroxidase (μmol pyrrogalloline $mg^{-1}$ protein $min^{-1}$) | 31.1 ± 23.8 |
| superoxide dismutase (unit SOD $mg^{-1}$ protein $min^{-1}$) | 13.0 ± 2.1 |
| ascorbate peroxidase (μmol ASC $mg^{-1}$ protein $min^{-1}$) | 13.0 ± 7.7 |
| nitrogen content (%) | 1.83 ± 0.08 |
| carbon content (%) | 44.48 ± 0.81 |
| $\delta^{13}$C (‰) | -30.61 ± 0.12 |
| $\delta^{15}$N (‰) | 3.31 ± 1.14 |

The contents of ASC and GSH varied more between monitoring stations (between-site variability) than between willows at the same monitoring station (inter-tree variability) (Table 5.4). This between-site variability was mainly related to the degree of shade (Table 5.4) with a negative relationship between shade and foliar ASC (Fig. 5.1a) and GSH (Fig. 5.1b).

For MDA, FLA, FRAP and POLY, the variability between the monitoring stations was low (Table 5.4). PCA1 was significantly related with MDA and POLY (Table 5.4): willows growing at sites with a higher $NO_2$ concentration and a lower $O_3$ concentration had a higher foliar MDA (22.0 nmol $g^{-1}$ FW) and POLY content (22.1 mg gallic acid $g^{-1}$ FW) compared to willows at sites with a higher $O_3$ and a lower $NO_2$ concentration (16.2 nmol MDA $g^{-1}$ FW and 16.3 mg gallic acid $g^{-1}$ FW) (Fig. 5.1c, e). PCA1 was also related with the between-site variability of FRAP (Table 5.4); FRAP increased with increasing $NO_2$ and decreasing $O_3$ concentrations (Fig. 5.1d).

The activities of the measured enzymes were characterized by a high variability between the willows at the same monitoring station rather than between the monitoring stations (Table 5.4). This pronounced between-site variability was not correlated with any explanatory variable used in the present study (Table 5.4).

### 5.3.2 Stable C and N isotopes

The mean foliar $\delta^{13}C$ and $\delta^{15}N$ values correlated significantly with the mean foliar C and N contents (Table 5.2, Table 5.3). Leaves at monitoring stations with a high degree of shade had a lower C and higher N content than leaves developed under high light (Table 5.4, Fig. 5.2a-b). For $\delta^{13}C$, the between-site variability was related with PCA1 and shade, where an increase of PCA1 (Fig. 5.2c) and shade (Table 5.4) decreased the foliar $\delta^{13}C$ value. Foliar $\delta^{15}N$ values were not related with soil $\delta^{15}N$ values, which ranged from -0.81‰ to -1.5‰.

Table 5.3: Spearman correlation coefficients between pairs of biochemical leaf characteristics [reduced ascorbate (ASC, µmol $g^{-1}$ FW), reduced glutathione (GSH, µmol $g^{-1}$ FW), malondialdehyde (MDA, nmol $g^{-1}$ FW), flavonoid (FLA, mg quercetin $g^{-1}$ FW), total antioxidant capacity (FRAP, µmol trolox $g^{-1}$ FW), polyphenols (POLY, mg gallic acid $g^{-1}$ FW), peroxidase(POX, µmol pyrrogalloline $mg^{-1}$ protein $min^{-1}$), superoxide dismutase (SOD, $mg^{-1}$ protein $min^{-1}$), ascorbate peroxidase (APX, µmol ascorbate $mg^{-1}$ protein $min^{-1}$), nitrogen content (N, %), carbon content (C, %), $\delta^{13}$C (‰), $\delta^{15}$N (‰)], a '*' indicates a significant difference at the 0.05 level

| | GSH | MDA | POLY | FLA | FRAP | POX | APX | SOD | N | C | $\delta^{15}$N | $\delta^{13}$C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ASC | 0.809* | -0.291 | 0.176 | 0.209 | -0.076 | -0.3 | 0.059 | -0.429 | -0.669 | 0.491 | 0.244 | 0.706* |
| GSH | | -0.224 | 0.209 | 0.056 | 0.179 | -0.203 | -0.079 | -0.479 | -0.579* | 0.488 | 0.253 | 0.675* |
| MDA | | | 0.768* | 0.499* | 0.585* | -0.274 | -0.25 | -0.121 | 0.268 | -0.132 | 0.144 | -0.483 |
| POLY | | | | -0.215 | 0.379 | -0.371 | -0.065 | -0.126 | -0.131 | 0.085 | 0.406 | -0.026 |
| FLA | | | | | 0.026 | 0.429 | 0.309 | -0.003 | 0.133 | 0.182 | -0.165 | -0.118 |
| FRAP | | | | | | -0.224 | -0.406 | -0.329 | 0.196 | 0.165 | 0.156 | -0.081 |
| POX | | | | | | | 0.118 | -0.026 | 0.592* | 0.182 | -0.465 | -0.193 |
| APX | | | | | | | | 0.441 | -0.153 | -0.344 | -0.021 | -0.049 |
| SOD | | | | | | | | | 0.141 | -0.288 | 0.103 | -0.29 |
| N | | | | | | | | | | -0.001 | -0.274 | -0.628* |
| C | | | | | | | | | | | 0.038 | 0.527* |

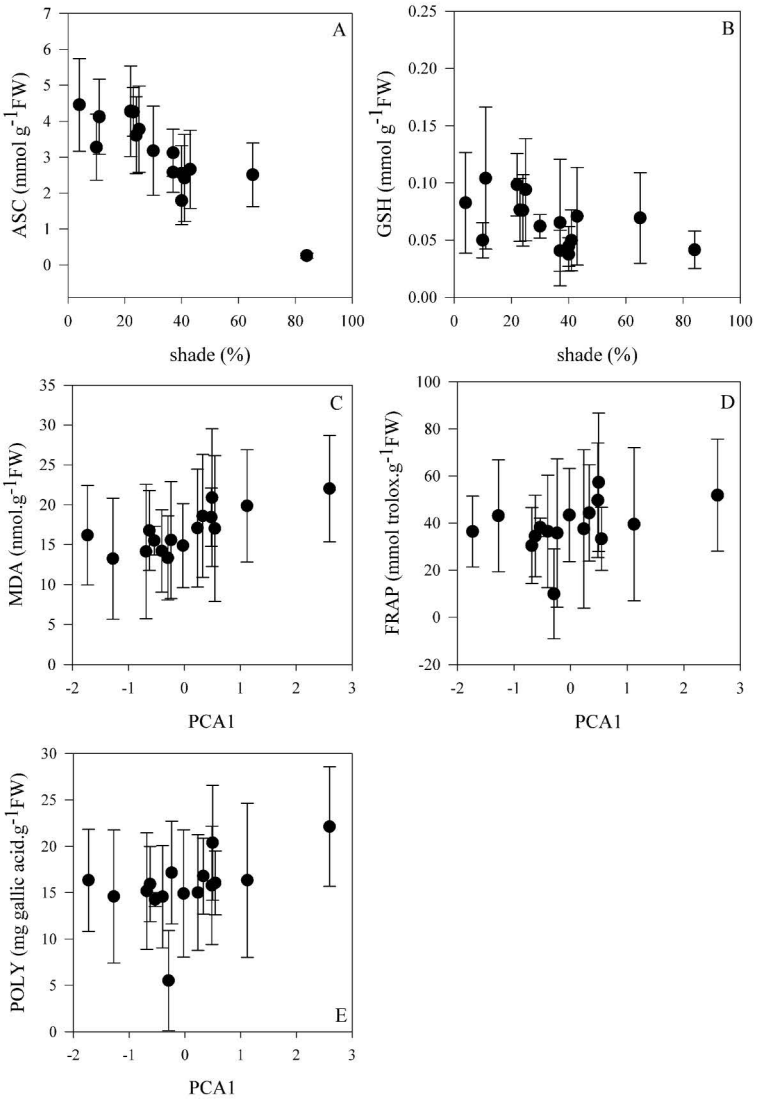


Figure 5.1: Mean and standard deviation (n = 16) of foliar (a) reduced ascorbate (ASC) and (b) reduced glutathione (GSH) of white willow at each monitoring station as a function of the degree of shade, (c) malondialdehyde (MDA), (d) polyphenols (POLY) and (e) total antioxidant capacity (FRAP) of white willow at each monitoring station as a function PCA1

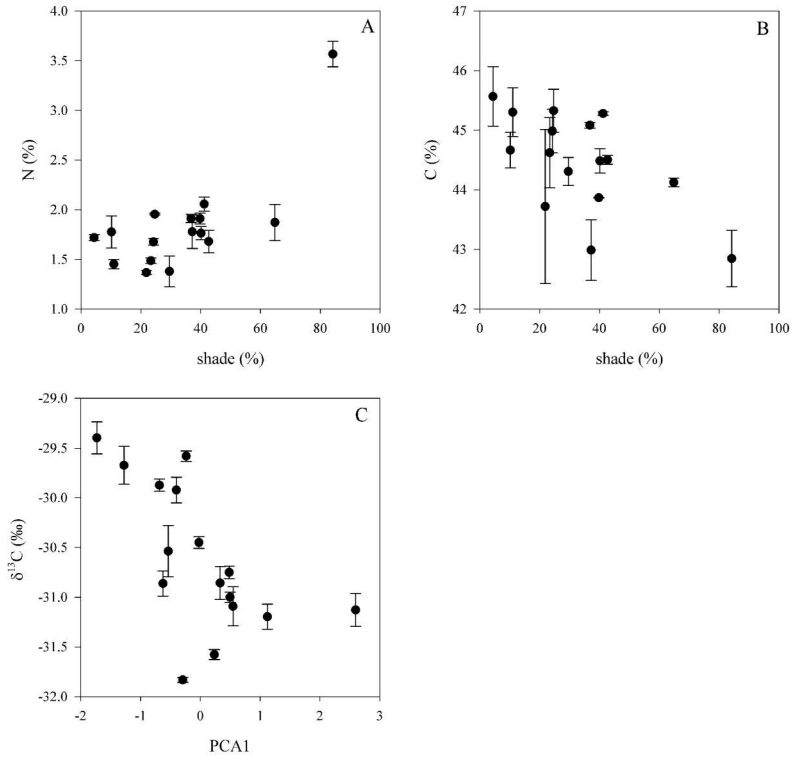


Figure 5.2: Mean and standard deviation of foliar (a) nitrogen content (N), (b) carbon content (C) as a function of the degree of shade and (c) $\delta^{13}$C of white willow at each monitoring station as a function of PCA1, respectively

Table 5.4: Between-site variability (i.e., the variability between the 16 monitoring stations; %), inter-tree variability (i.e., the variability between the 12 stem cuttings at the same monitoring station; %) of biochemical leaf characteristics, explained between-site variability (%). For the explanatory variables of the linear mixed models the significance and t, F or chi-square value are given; [a] explained between-site variability by PCA1 and shade; n/a: not applicable

| Characteristic | Between-site variability | Inter-tree variability | Explanatory variable | Explained between-site variability |
|---|---|---|---|---|
| ASC | 45 | 36 | Shade ($p < 0.001$, $t = -6.13$) | > 50 |
| GSH | 59 | 27 | Shade ($p < 0.001$, $t = -6.41$) | > 50 |
| MDA | 3 | 4 | PCA1 ($p < 0.001$, $t = 3.63$) | > 90 |
| FlA | < 0.1 | 0.2 | None ($p = 0.257$, $F = 1.26$) | n/a |
| FRAP | < 0.1 | 35 | PCA1 ($p = 0.021$, $t = 2.34$) | 58 |
| POLY | 9.5 | n/a | PCA1 ($p = 0.009$, $t = 3.02$) | > 90 |
| POX | 15 | 30 | None ($p = 0.520$, $\chi^2 = 10.11$) | n/a |
| SOD | < 0.1 | 19 | None ($p = 0.638$, $F = 0.802$) | n/a |
| APX | 0.2 | 65 | None ($p = 0.347$, $F = 1.13$) | n/a |
| N | 88 | n/a | Shade ($p = 0.0013$, $t = 4.01$) | 50 |
| $\delta^{13}C$ | 87 | n/a | PCA1 ($p = 0.011$, $t = -3.02$)<br>Shade ($p = 0.001$, $t = -4.16$) | 86[a] |
| $\delta^{15}N$ | 87 | n/a | None ($p = 0.849$, $F = 0.335$) | n/a |
| C | 88 | n/a | Shade ($p = 0.009$, $t = -2.99$) | > 90 |

## 5.4 Discussion

### 5.4.1 Lipid peroxidation and antioxidant system

Pollutants have the potential to disrupt plant-biochemical processes after absorption through stomata or the cuticle (e.g., Furlan et al. 2010). They dissolve in the extracellular fluid and disrupt the cellular homeostasis, leading to an enhanced production of ROS (Mansfield and FreerSmith 1981, Mittler 2002). ROS are highly toxic for plants and can cause, for example, peroxidation of poly-unsaturated fatty acids of the cell membrane (Wannaz et al. 2003, Rai and Agrawal 2008). This peroxidation leads to the accumulation of end products, such as MDA (Mehlhorn et al. 1991). In this study, accumulation of MDA and thus peroxidation of the cell membrane occurred at monitoring stations with a high atmospheric $NO_2$ concentration (Fig. 5.1c). Chen et al. (2010) also observed an increase in lipid peroxidation of one-year-old *Cinnamomum camphora* L. seedlings exposed to high atmospheric $NO_2$ concentrations. Furthermore, MDA can trigger an enhanced production of free radicals, which will react with proteins, DNA and membrane lipids to cause reduced photosynthesis, electrolyte leakage and accelerated senescence (Sharma and Davis 1997).

The non-enzymatic antioxidant system can counteract these adverse effects of ROS on sensitive cellular components, such as membrane lipids, by removing and/or neutralizing ROS (Mittler 2002). Key components of the non-enzymatic antioxidant network are leaf ASC and GSH, which are involved in the ASC-GSH cycle to eliminate $H_2O_2$ and $O_2{}^{-1}$ radicals (Mittler 2002, Galant et al. 2011). Levels of leaf ASC and GSH are believed to increase as a consequence of exposure to air pollutants (Madamanchi et al. 1991, Tiwari and Agrawal 2011), but decreases (Bermadinger et al. 1990) and no alterations (Hausladen et al. 1990, Klumpp et al. 2000, Tausz and Grill 2000) are also observed. In this study, on the one hand, the level of leaf ASC and GSH was not linked to the ambient $NO_2$ and/or $O_3$ concentration. It is possible that a damaged photosynthetic apparatus, due to air pollution stress, leads to the absence of reducing energy that is necessary for the ROS-removing by the ASC-GSH cycle (Mittler 2002), or that the low level air pollution did not cause the initiation of ASC and/or GSH production. To verify this hypothesis, measurements of chlorophyll fluorescence are appropriate. On the other hand, the level of leaf ASC and GSH was negatively affected by the degree of shade (Fig. 5.1a-b), which has also been found by other studies (e.g., Noctor et al. 1997, Smirnoff and Wheeler 2000, Tausz and Grill 2000). Noctor et al. (2012) stated that light-induced changes in leaf GSH content are partly the result of light-

dependent changes in rates of GSH breakdown or export, the restriction of the conversion of $\gamma$-glutamylcysteine to GSH and a decreased availability of glycine. According to Yabuta et al. (2007) mainly GDP-D-mannose pyrophosphorylase, L-galactose 1-P phosphatase and L-galactono-1,4-lactone dehydrogenase are down-regulated under shade, leading to a reduced level of leaf ASC (Logan et al. 1996, Massot et al. 2012). Not only light quantity, but also light quality (red/far red ratio) plays an important role in leaf ASC and GSH synthesis as well as the regeneration of ASC and GSH from its oxidized forms (Bartoli et al. 2009).

Other components of the non-enzymatic antioxidant system are the POLY, which can be divided into three groups: gallic acids derivatives (GA), hydroxycinnamic acid derivatives (HCA) and FLA. Each group can be further divided into various classes, such as flavones, flavanes, flavonols, catechins and anthocyanidins for the FLA group (Amic et al. 2003) and subclasses, such as quercetin, myricetin, larycitrin and syringetin for the flavonol class. Foliar contents of total phenols have been found to increase with increasing distance to the air pollution source (Loponen et al. 1998, Giertych et al. 1999), but decreases (Krywult et al. 1996, Pasqualini et al. 2003) and no alterations (Robles et al. 2003) are also reported. Furthermore, detailed studies have also demonstrated that individual POLY groups, classes and subclasses can respond differently to air pollution (Robles et al. 2003). For example, Loponen et al. (2001) found an increase of GA, a decrease of FLA and no alteration of HCA due to air pollution and Robles et al. (2003) found a decrease of anthocyanidins and an increase of flavonols with increasing atmospheric $SO_2$ and $O_3$ concentration. In our study, total POLY contents increased with an increasing $NO_2$ concentration (Fig. 5.1d), indicating the activation of a defense mechanism against the oxidative stress caused by atmospheric $NO_2$. The increase of the total POLY contents was not caused by an increase of FLA, since FLA was not influenced by air pollution, but can be due to an increase of the GA and/or HCA content. More detailed research, which includes measurements of the different phenolic groups, classes and subclasses, is necessary to understand the impact on air pollution on total phenols.

In general, the total non-enzymatic antioxidant capacity (FRAP) increased with an increase of PCA1, i.e., the antioxidant capacity was higher at monitoring stations with a higher mean $NO_2$ concentration. This relationship was probably due to the relation between the POLY and PCA1. It must also be noted that we only took account of the abiotic factors shade, vapor pressure deficit and air pollution, while several other, non-measured biotic and abiotic factors can also lead to oxidative stress, which is suggested by the

inter-tree variability. Loponen et al. (1998) also found a high among-tree variability in phenols, which made it difficult to find consistent differences between phenols of trees in polluted versus control areas.

Besides the non-enzymatic antioxidant system, an enzymatic antioxidant system, consisting of, for example, SOD, APX and POX, can also counteract the adverse effects of ROS on sensitive cellular components (Mittler et al. 2004). An increased activity of SOD, which dismutates superdioxide into $H_2O_2$ and oxygen, and of POX and APX, which further reduce $H_2O_2$ into water and oxygen, is an important defense mechanism of plants in response to air pollution (Sharma and Davis 1997, Pucinelli et al. 1998, Kammerbauer and Dick 2000, Mittler 2002, Li 2003). However, POX is considered to be a general indicator for oxidative stress (Roitto et al. 1999), instead of a specific indicator of a single air pollutant, which could possible explain the inability to link PCA1 to POX activity. Moreover, it is possible that the response of the measured enzymes to air pollutants is modified by other internal or external factors (Roitto et al. 1999), leading to the absence of a link between air pollution and SOD, APX and POX activity in this study. Other enzymes than SOD, APX and POX can be responsible for the enzymatic defense against air pollution stress. Catalase (CAT), for example, which is present in the peroxisomes, is also responsible for the removal of excess ROS during stress (Mittler 2002). It is possible that air pollution stress suppressed the CAT production, which in turn induced APX and POX to compensate for the loss of CAT at monitoring stations with a high $NO_2$ concentration (Mittler 2002).

### 5.4.2 Stable C and N isotopes

The $\delta^{13}C$ has been used to examine ecological, biogeochemical and physiological processes related to C cycles (Farquhar et al. 1989), while the $\delta^{15}N$ has been used for gathering information about N deposition and plant's N availability (Robinson 2001). In addition, changes in $\delta^{13}C$ are frequently used as early warning indicators of air pollution stress (e.g., $SO_2$, $O_3$ and $NO_x$) (Saurer et al. 1995, Siegwolf et al. 2008, Kwak et al. 2009, Battiplagia et al. 2010, Liu et al. 2010). In our study, significantly lower $\delta^{13}C$ values in leaf tissues were found at monitoring stations with a high atmospheric $NO_2$ but low atmospheric $O_3$ concentration (e.g., Voorhaven; Table 5.1), with an average depletion of 2‰ over the PCA1 range (Fig. 5.2c), while no effect of air pollution on $\delta^{15}N$ was found (Table 5.4). Balasooriya et al. (2009) reported similar results; in more urbanized and industrial land use classes lower foliar $\delta^{13}C$ values were observed for *Taraxacum officinalis*, with an average depletion of 2‰, but no alterations of $\delta^{15}N$ values

were observed. The $\delta^{13}C$ value of leaf tissues is related to changes in stomatal conductance (Farquhar et al. 1982) and/or biochemical characteristics affecting photosynthesis (Dawson et al. 2002). In turn, discrimination against $^{13}C$ by the carboxylating enzyme is linked to photosynthesis via the ratio of the internal $CO_2$ concentration to the atmospheric $CO_2$ concentration (Dawson et al. 2002). As a consequence, a lower rate of net photosynthesis (Farquhar et al. 1989, Matyssek et al. 1992) or a decreased carboxylation efficiency due to the degradation of chlorophyll (Shan 1998), in case of high air pollution, can lead to an increased discrimination against $^{13}C$ (Warren and Dreyer 2006). This increased discrimination can possibly explain the decrease in $\delta^{13}C$ due to the high (stressful) $NO_2$ concentration found in this study. Another possible explanation of the $^{13}C$ depletion can be found in the assimilation of $\delta^{13}C$ depleted ambient air in areas with high road traffic and domestic heating (Balasooriya et al. 2009). In accordance, Kwak et al. (2009) also interpreted the decreased $\delta^{13}C$ values in their study as a reflection of the assimilation of $^{13}C$ depleted atmospheric $CO_2$. In addition, the higher anthropogenic $CO_2$ emission from various fossil-fuel combustion sources in the urban atmosphere can enhance C assimilation of urban plants, by which discrimination against $^{13}C$ in leaf cells would increase (O'Leary 1981). Discrimination against $^{13}C$ has also been observed to vary in response to irradiance (Zimmerman and Ehleringer 1990), due to the direct effect of light intensity on the leaf intercellular $CO_2$ concentration and/or stomatal conductance (Farquhar et al. 1982, Pfitsch and Pearcy 1992, Yakir and Israeli 1995, D'Allessandro et al. 2006, Hu et al. 2012). In the present study, $\delta^{13}C$ was more negative under low-light conditions, with a mean $^{13}C$ depletion of 2‰. Changes in $\delta^{13}C$ value probably reflected changes in C and N leaf content to light, since $\delta^{13}C$ and C and N were significantly correlated (Table 5.3).

In contrast to $\delta^{13}C$, $\delta^{15}N$ of white willow was not linked to the ambient air quality, confirming the statement of Dawson et al. (2002) that it is difficult to use $\delta^{15}N$ as a tool for understanding the relationship between plants and ambient air pollution. In early studies, $\delta^{15}N$ of leaf tissues was assumed to reflect the $\delta^{15}N$ of the N-form most abundantly taken up by that plant. However, it is now clear that such an interpretation is not true, since the natural abundance of $^{15}N$ in plants reflects the net effect of a range of processes (assimilation, translocation, mineralization, N loss). The foliar $\delta^{15}N$ value can also be affected by changes in plant demand as is the case with C, e.g., injurious effects of air pollution on stomatal aperture can result in a reduced discrimination against heavier C isotopes (Norra et al. 2005). In our study, positive foliar $\delta^{15}N$ values were observed at all monitoring sta-

tions, probably as a consequence of local conditions such as traffic density, mean distribution of heavy- and light-duty engines, and average combustion regimes in the engines, as stated by Ammann et al. (1999). For example, diesel particles are enriched by $^{15}N$ compared to fuel-oil particles (Widory 2007), $NO_x$ emitted by coal-fired power stations are enriched by $^{15}N$ compared to $NO_x$ emitted by vehicles (Heaton 1990) and agricultural N emissions are relatively depleted in $^{15}N$ (Heaton 1986). In addition, differences in leaf $\delta^{15}N$ between the monitoring stations did not result from differences in soil $\delta^{15}N$, since willows were planted in uniform potting soil and all soil $\delta^{15}N$ values were in a anarrow range.

## 5.5 Conclusions

This study demonstrates that ambient $NO_2$ concentration interrupts the normal (biochemical) functioning of plants, by enhancing the production of ROS. The excess ROS lead to the peroxidation of membrane lipids, causing increased amounts of MDA, and activated the antioxidant system. Only POLY content was higher at monitoring stations with a high amount of MDA, while the content of ASC, GSH and FLA and the activity of enzymes were not related to the atmospheric $NO_2$ concentration. Based on our results, it is not clear whether this activation of the antioxidant system will protect the plant from further damage, and, therefore, measurements of ROS can be highly interesting to obtain information about the rate of ROS scavenging. At monitoring stations with a high atmospheric $NO_2$ concentration, also $\delta^{13}C$ was more negative compared to monitoring stations with a low atmospheric $NO_2$ concentration. A lower rate of net photosynthesis and stomatal conductance, due to a high atmospheric $NO_2$ concentration, and/or assimilation of $^{13}C$ depleted ambient air from excess fossil fuel sources were presented as possible hypotheses for the changes of $\delta^{13}C$. To support one of these hypotheses, measurements of photosynthesis (by chlorophyll fluorescence) and/or stomatal conductance (by nail varnish method, §2.2.2.3) in combination with $\delta^{13}C$ of atmospheric $CO_2$ are necessary.

In conclusion, active biomonitoring with MDA, POLY and $\delta^{13}C$ of white willow seems promising. However, preference is given to the use of MDA as an early warning indicator for ambient air quality, since MDA measurements are (i) easy to carry out, (ii) less expensive compared to stable isotope measurements and (iii) independent of shade, which allows to choose sample sites with a different degree of shade and to take leaves from both shaded and unshaded positions.

# 6

# The species-dependent response of white willow, northern red oak and Scots pine to ambient air quality

*After: Wuytack, T., Samson, R., Wuyts, K., Adriaenssens, S., Kardel, F., Verheyen, K.. Do leaf characteristics of white willow (Salix alba L.), northern red oak (Quercus rubra L.) and Scots pine (Pinus sylvestris L.) respond differently to ambient air pollution and other environmental stressors? Submitted to Journal of Environmental Monitoring*

This study assessed the effect of ambient air pollution on leaf characteristics of white willow, northern red oak and Scots pine. Willow, oak and pine saplings were planted at sixteen locations in Belgium, where atmospheric $NO_2$, $O_3$, $SO_2$ and $PM_{10}$ concentrations were continuously measured. The trees were exposed to ambient air during six months (April - September 2010), and, thereafter, specific leaf area (SLA), stomatal resistance ($R_S$), leaf area fluctuating asymmetry (FAA), contact angle (CA), relative chlorophyll content and maximum photochemical efficiency of photosystem II ($F_v/F_m$) were measured. Leaf characteristics of willow, oak and pine were differently related to the ambient air pollution, indicating a species-

dependent response. Willow had a higher SLA and $R_S$, pine had a higher SLA and $F_v/F_m$ and oak had a higher $F_v/F_m$ and a lower FA at monitoring stations with higher atmospheric $NO_2$ and lower atmospheric $O_3$ concentrations. Willow and oak seem to be the most suitable species to use in active biomonitoring studies, while Scots pine is not recommended, since planting is difficult due to drought sensitivity of roots and measuring of leaf characteristics on needles is time-consuming.

## 6.1 Introduction

The extent to which plant characteristics are affected by air pollutants is species-specific (Larcher 2003), eliciting different changes for sensitive species or even no changes for tolerant species. Bassin et al. (2009) demonstrated that specific leaf area (SLA) of *Ligustrum lucidum* and *Leontodon helveticus* increased, while Tiwari et al. (2006) demonstrated that SLA of carrot plants decreased as a result of increased air pollution. Conflicting results are also published for fluctutaing asymmetry (FA) (Velickovic and Perisic 2006, Kozlov et al. 2009) and leaf wettability (Kardel et al. 2012). As a consequence, the effectivity of an air quality biomonitoring study stands or falls with the choice of the used species. In Chapter 4-5, we evaluated only the potential of white willow to monitor the ambient air quality, and, therefore, we wanted to investigate which of the three contrasting tree species, white willow (fast growing, broad-leaved deciduous), northern red oak (more slowly growing, broad-leaved deciduous) and Scots pine (evergreen conifer) is most suitable to use in biomonitoring studies. We hypothesized that a fast-growing species, with a high atmospheric interaction and thus also a high uptake of air pollutants, and a coniferous species, with a small boundary layer and thus a high interaction with the atmosphere, will have a more pronounced response to ambient air quality compared to a slow-growing deciduous species.

## 6.2 Materials and methods

### 6.2.1 Study area and experimental design

The study was performed in the near vicinity of the sixteen air quality monitoring stations in Belgium (§1.1.3.2, Fig. 1.3, Table 4.1), were the concentration of atmospheric pollutants is continuously measured. In addition, meteorological data is obtained from weather stations near these monitor-

ing stations. The concentration of the atmospheric pollutants, air temperature (T, °C), relative air humidity (RH, %), shade and the site-specific value for air quality (PCA1) are given in Table 4.2 (see second in-leaf season).

White willow, northern red oak and Scots pine were used as active biomonitors. Willow is a fast growing deciduous species with a high gas exchange rate, due to a high stomatal conductance, that results in an intensive interaction with the atmosphere. Also, the use of clonal stem cuttings gives the advantage that phenotypic variation is likely to be a reflection of the experienced environment rather than genotypic differences. The use of northern red oak and Scots pine is based on the multiple use of these species in biomonitoring studies and the wide-spread abundance in the region of Flanders. Stem cuttings of willow were provided by De Vos 'Salix', a company specialized in cultivation and processing of *Salix* spp., and saplings of oak and pine were obtained from a local nursery.

At each monitoring station, four cuttings of willow (length: 18 cm) were planted in 3.5 $dm^3$ pots with homogeneous potting soil as described in §4.2.2. In addition, also five 3-year old saplings of oak and 2-year old saplings of pine were planted at each monitoring station in April 2010. Saplings of oak and pine were planted in, respectively, 5 $dm^3$ and 3.5 $dm^3$ pots with homogeneous potting soil. Since rooting volumes were not 'fenced off' with a porous membrane, root expansion outside the pots could occur through the holes in the bottom. Plants were spaced so as to minimize shading between plants. To avoid water deficiency, a semi-automatic water supply system was used (see §4.2.2). To counteract snail herbivory, copper tape was attached around the pots.

In September 2010, willow, oak and pine were harvested and several leaf characteristics were determined. Due to mortality, which is a side effect of working with living materials in real conditions, less than five (but at least two) Scots pines remained by the time of harvesting at most of the monitoring stations. At the monitoring stations 'Aarschot' and 'Veurne' no pines could be sampled.

## 6.2.2 Data acquisition

### 6.2.2.1 Leaf characteristics

For willow and oak, we randomly collected 20 fully developed and undamaged leaves to calculate leaf area fluctuating asymmetry (FAA) and SLA ($cm^2\ g^{-1}$) at each monitoring station, as described in Chapter 2 (see §2.2.2.2). For pine, we collected ten fully developed and undamaged nee-

dle fascicles at each monitoring station to calculate the projected leaf area (PLA) and needle asymmetry. Each needle per fascicle was mounted on paper in such a way that the needle was straightened and pushed flat. Length, width and thickness were measured by using a digital caliper (accuracy 0.01 mm) and weighed after oven-drying (at 70°C for 48 h). A needle was assumed to be an ellipsoid, and, therefore, PLA ($cm^2$) was calculated by using Eq. 6.1 (Sellin 2000).

$$PLA = \frac{\pi \times L \times D_2^2}{4 \times \sqrt{D_1^2 + D_2^2}} \tag{6.1}$$

with L the length (cm), $D_2$ the thickness (cm) and $D_1$ the width of a needle (cm). SLA was defined as the ratio of PLA to the needle biomass (g). The asymmetry of a needle fascicle was determined as the ratio of the difference in needle length between the two needles to the average needle length (Kozlov and Niemela 1999).

For illustration and interpretation purposes, leaf cross-sections were taken from willow (n = 10), oak (n = 10) and pine (n = 5) as described in Chapter 4 (see §4.2.3.2). For willow and oak, palisade ($R_p$, μm) and spongy parenchyma thickness ($R_{sp}$, μm) and total leaf thickness were measured and the coefficient of palisadeness (K, %) was calculated as the ratio of $R_p$ to the thickness of the mesophyll tissue ($R_p + R_{sp}$) (Dineva 2006).

For willow, stomatal imprints were made on the adaxial leaf side of ten fully developed and undamaged leaves at each monitoring station. For oak, which has hypostomatous leaves, stomatal imprints were made at the abaxial leaf side of ten fully developed and undamaged leaves at each monitoring station. For pine, which has amphistomatous needles, abaxial and adaxial stomatal imprints were made of five needles at each monitoring station. Stomatal imprints were made prior to harvesting according to the method described in Chapter 2 (see §2.2.2.3) and stomatal density (SD, $mm^{-2}$), stomatal pore surface (SPS, $\mu m^2$) and stomatal resistance ($R_S$, s $m^{-1}$) were calculated (see §2.2.2.3). However, due to the poor quality of the stomatal imprints of pine, only SD could be determined.

The leaf $F_v/F_m$ measurements were performed in situ as described in Chapter 4 (see §4.2.3.5). At each monitoring station, 15 leaves of willow and oak were randomly selected for the measurements. For pine, several needles were placed next to each other between transparent tape layers, making it possible to perform chlorophyll fluorescence measurements on needles (n = 5). To take the noise of the transparent tape into account, $F_v/F_m$ of two

transparent tapes was measured and subtracted from the other measurements.

Finally, leaf wettability was measured as the contact angle (CA) of standardized water droplets (7 µl for willow and oak, 3 µl for pine) with the leaf surface, as described in Chapter 4 (see §4.2.3.4). At each monitoring station, shoots or branches were placed in water-filled tubes and immediately transported to the laboratory, where leaves and needles were carefully excised to avoid wax abrasion. For willow and oak, two segments of the left and two segments of the right lamina side were excised from 20 leaves and mounted, abaxial and adaxial surface uppermost, onto glass slides using double sided tape. For pine, two segments were excised from each needle of ten needle fascicles and mounted, abaxial and adaxial surface uppermost, onto glass slides using double sided tape.

#### 6.2.2.2 Statistical analysis

Because of the hierarchical nature of the data, we used linear mixed models to relate the leaf characteristics to the set of explanatory variables, as described in Chapter 4 (see §4.2.4). For willow, shoot level was nested within stem cutting, which was nested within monitoring station, while for oak and pine, tree level was nested within monitoring station. Air quality (PCA1), VPD and shade and their first order interaction with air pollution were used as explanatory variables to analyze the leaf characteristics. For the stomatal characteristics, also SLA was used as an explanatory variable (see §4.2.4).

## 6.3 Results

Mean values of all measured leaf characteristics for willow, oak and pine are given in Table 6.1 and the results of the mixed model, as well as the between-site (i.e., the variability of leaf characteristics between the monitoring stations), inter-tree (i.e., the variability of leaf characteristics between stem cutitings at the same monitoring station) and intra-shoot (i.e., the variability of leaf characteristics between leaves of the same stem cutting) variability are given in Table 6.2. RCC of oak and CA of willow, oak and pine of the left and right leaf side were significantly correlated ($p < 0.001$, $r = 0.917$, $N = 320$), and, therefore, mean RCC and CA were calculated and used for further analyses. The degree of shade had a significant influence on the SLA of willow, oak, and pine, the $R_S$ and RCC of oak, $F_v/F_m$ of willow and oak and the adaxial CA ($CA_{ad}$) of willow (Table 6.2). To minimize the confounding effect of shade, we focused our anal-

ysis of these leaf characteristics on the monitoring stations with less than 80% shade. Re-analyzing the data showed no differences in the obtained results, except for SLA. PCA1, which is positively correlated with the atmospheric $NO_2$ concentration and negatively with the $O_3$ concentration, explained 52% and 56% of the between-site variability of SLA of, respectively, willow and pine. In addition, PCA1 was correlated with FAA of oak, $R_S$ of willow and $F_v/F_m$ of pine (Table 6.2).

Table 6.1: Mean of specific leaf area (SLA, $cm^2\ g^{-1}$), leaf area fluctuating asymmetry (FAA, -), stomatal resistance ($R_S$, $s\ m^{-1}$), maximum photochemical efficiency of photosystem II ($F_v/F_m$, -), relative chlorophyll content (RCC, -), stomatal density (SD, $mm^{-2}$) and contact angle (CA, °) of willow, oak, and pine for each monitoring station; n/a: not applicable

| | Willow | | | | | Oak | | | | | | | Pine | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Station | SLA | FAA | $R_S$ | $F_v/F_m$ | CA | FAA | SLA | $R_S$ | $F_v/F_m$ | $CA_{ab}$ | $CA_{ad}$ | RCC | SLA | FA | $SD_{ad}$ | $SD_{ab}$ | $F_v/F_m$ | $CA_{ad}$ | $CA_{ab}$ |
| Aarschot | 125.67 | 0.044 | 77.76 | 0.83 | 65.81 | 0.03 | 161.89 | 16.99 | 0.82 | 114.25 | 79.63 | 11.91 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| Berendrecht | 150.06 | 0.002 | 105.15 | 0.8 | 70.76 | 0.03 | 158.64 | 15.15 | 0.77 | 107.55 | 78.73 | 10.61 | 42.43 | 0 | 101.1 | 127.89 | 0.8 | 67.92 | 58.73 |
| Bergen | 144.47 | 0.059 | 111.57 | 0.84 | 74.87 | 0.04 | 161.9 | 13 | 0.78 | 106.82 | 76.39 | 12.85 | 53.86 | -0.004 | 94.82 | 123.29 | 0.82 | 68.54 | 69.16 |
| Borgerhout | 121.89 | 0.056 | 103.72 | 0.84 | 57.07 | 0.03 | 169.24 | 18.61 | 0.79 | 104.59 | 83.07 | 9.6 | 45.68 | -0.048 | 123.92 | 132.5 | 0.85 | 86.2 | 75.48 |
| Charleroi | 156.71 | 0.024 | 93.42 | 0.79 | 64.7 | 0.05 | 199.81 | 17.04 | 0.82 | 107.77 | 67.66 | 15.19 | 52.41 | 0.002 | 109.89 | 88.75 | 0.83 | 68.14 | 60.47 |
| Corroy | 111.64 | 0.027 | 70.08 | 0.83 | 68.58 | 0.03 | 153.72 | 14.79 | 0.76 | 80.34 | 69.54 | 11.78 | 39.45 | -0.01 | 102.78 | 144.85 | 0.81 | 61.95 | 61.6 |
| Dourbes | 108.33 | 0.032 | 72.13 | 0.79 | 65.54 | 0.06 | 147.04 | 16.79 | 0.79 | 78.22 | 63.52 | 10.14 | 32.28 | 0.013 | 96.71 | 107.8 | 0.75 | 62.12 | 62.99 |
| Engis | 142.38 | 0.051 | 80.99 | 0.83 | 71.62 | 0.04 | 158.87 | 14.24 | 0.76 | 89.44 | 60.14 | 10.17 | 42.92 | -0.018 | 92.73 | 94.19 | 0.77 | 53.14 | 52.36 |
| Hasselt | 115.72 | 0.021 | 87.22 | 0.84 | 63.97 | 0.04 | 137.38 | 14.64 | 0.79 | 105.92 | 90.7 | 8.93 | 44.13 | -0.016 | 98.38 | 114.5 | 0.79 | 65.78 | 57.48 |
| Mendonk | 133.83 | 0.017 | 90.71 | 0.81 | 68.34 | 0.03 | 161.26 | 15.18 | 0.8 | 96.93 | 80.47 | 11.18 | 44.51 | 0.001 | 106.12 | 109.89 | 0.83 | 68.32 | 61.18 |
| Sinsin | 120.18 | 0.035 | 69.95 | 0.81 | 71.36 | 0.05 | 140.05 | 13.46 | 0.76 | 94.35 | 66.21 | 11.98 | 36.31 | 0.008 | 105.91 | 123.92 | 0.74 | 63.77 | 65.9 |
| Ukkel | 115.58 | 0.059 | 86.08 | 0.86 | 76.22 | 0.05 | 157.45 | 15.47 | 0.75 | 85.6 | 76.64 | 7.6 | 29.27 | -0.004 | 108.22 | 131.45 | 0.78 | 61.55 | 59.77 |
| Veurne | 148.85 | 0.018 | 96.98 | 0.79 | 65.72 | 0.03 | 156.96 | 16.03 | 0.84 | 84.56 | 73.05 | 13.04 | n/a | n/a | n/a | n/a | n/a | n/a | n/a |
| Voorhaven | 180.38 | 0.005 | 110.78 | 0.85 | 76.93 | 0.03 | 154.04 | 13.63 | 0.75 | 94.85 | 68.54 | 11.5 | 47.74 | -0.005 | 86.03 | 117.01 | 0.79 | 69.28 | 81.22 |
| Zwevegem | 144.99 | 0.02 | 81.67 | 0.82 | 67.62 | 0.04 | 158.15 | 16.38 | 0.82 | 107.17 | 75.06 | 13.47 | 35.14 | 0.012 | 122.03 | 139.61 | 0.82 | 72.4 | 69.31 |
| Schoten | 237.42 | 0.031 | 125.9 | 0.86 | 83.85 | 0.05 | 237.73 | 19.27 | 0.82 | 73.87 | 52.12 | 25.44 | 86.02 | 0.005 | 106.54 | 132.29 | 0.8 | 74.75 | 63.73 |

Table 6.2: The percentage of variation at each level that is explained by the optimal random model for all response variables [SLA ($cm^2$ $g^{-1}$, FA (-), $R_S$ ($s\ m^{-1}$), SPS ($\mu m^2$), SD ($mm^{-2}$), RCC (-), $F_v/F_m$ (-), CA (°)]; the significant contribution (indicated by the p- and t-value) of the explanatory variables [shade (%), PCA1, SLA and VPD (Pa)] that are retained in the fixed effect model as well as the percentage of between-site variability that is explained by the explanatory variables; the p- and likelihood ($\chi^2$) value for the anova-test to compare the fixed effect model with and without all explanatory variables; n/a: not applicable

| | | Between-site variability (%) | Inter-tree variability (%) | Intra-shoot variability (%) | Explanatory variable | Explained between-site variability (%) |
|---|---|---|---|---|---|---|
| SLA | Willow | 44 | 4 | 18 | shade (p = 0.004, t = 3.46) | 80 |
| | Oak | 10 | 23 | n/a | shade (p < 0.001, t = 4.38) | 76 |
| | Pine | 47 | n/a | n/a | shade (p = 0.0016, t = 4.062) | 64 |
| FA | Willow | 6 | 5 | 15 | none (p = 0.538, $\chi^2$ = 7.96) | n/a |
| | Oak | 3 | n/a | n/a | PCA1 (p = 0.0262, t = -2.23) | 67 |
| | Pine | < 0.1 | n/a | n/a | none (p = 3.063, F = 1.195) | n/a |
| $R_S$ | Willow | 17 | 12 | n/a | PCA1 (p < 0.001, t = 4.96) | 90 |
| | Oak | 12 | 32 | n/a | shade (p = 0.0024, t = 3.865) | 93 |
| SPS | Willow | 12 | n/a | n/a | PCA1:VPD (p = 0.0031, t = -3.687) | 90 |
| | Oak | 18 | 42 | n/a | none (p = 0.259, $\chi^2$ = 13.55) | n/a |
| SD | Willow | 29 | 26 | n/a | SLA (p = 0.002, t = -3.99)<br>VPD (p = 0.0066, t = -3.27) | 90 |
| | Oak | 6 | 47 | n/a | none (p = 0.113, $\chi^2$ = 16.816) | n/a |
| | Pine | 33 | n/a | n/a | none (p = 0.239, $\chi^2$ = 11.57) | n/a |
| RCC | Oak | 52 | 26 | n/a | shade (p = 0.002, t = 3.847) | 59 |
| $F_v/F_m$ | Willow | 43 | 17 | n/a | shade (p = 0.0004, t = 3.52) | 80 |
| | Oak | 56 | 4 | n/a | shade (p = 0.002, t = 3.21) | 27 |
| | Pine | 15 | n/a | n/a | PCA1 (p = 0.011, t = 3.00) | 64 |
| CA | Willow | < 0.1 | n/a | n/a | shade (p = 0.048; t = -2.17) | 50 |
| | Oak | 27 | n/a | n/a | none (p = 0.697, $\chi^2$ = 8.18) | n/a |
| | Pine | 23 | n/a | n/a | none (p = 0.106, $\chi^2$ = 13.18) | n/a |

## 6.4 Discussion

### 6.4.1 The response of willow, oak and pine to ambient air quality

Leaf characteristics of willow, oak and pine were affected by the ambient $NO_2$ and $O_3$ concentration (PCA1), but, the degree and nature of the response is species-dependent. Since PCA1 is positively correlated with the $NO_2$ concentration and negatively with the $O_3$ concentration, it is rather difficult to determine which of the atmopsheric pollutant ($NO_2$ or $O_3$) is most important in controlling the response of leaf characteristics or whether the change in leaf characteristics is the result of a synergistic or antagonistic interactions between atmospheric $NO_2$ and $O_3$ concentrations. Tiwari et al. (2006) demonstrated that, although the individual $SO_2$, $NO_x$ and $O_3$ concentrations were below the reference value for plant injury, the combined effect of the atmospheric pollutants seems to act synergistically and decreases plant growth. An antagonistic interaction effect of $NO_2$ and $O_3$ on the growth of spring rape was found by Adaros et al. (1991), and, similarly, Jäger et al. (1992) showed that atmospheric $O_3$ exposure counteracted the positive effect of relatively low $NO_2$ levels. In conclusion, we can only make assumptions concerning the effect of air pollution on the leaf characteristics and conclude that the level of $NO_2$ and/or $O_3$ caused changes in leaf characteristics.

Willow and pine had a higher SLA at monitoring stations with a high atmospheric $NO_2$ concentration, compared to the monitoring stations with a high atmospheric $O_3$ concentration, while SLA of oak seemed to be insensitive to $NO_2$ and/or $O_3$. In addition, willow and pine had, respectively, a higher $R_S$ and a higher $F_v/F_m$ at monitoring stations with a high atmospheric $NO_2$ concentration, while $R_S$ of oak, SD of pine and $F_v/F_m$ of willow did not respond to $NO_2$ and/or $O_3$. Stressful conditions lead to an increase of $R_S$ for minimizing the uptake of pollutants, optimizing the $CO_2$ uptake and reducing the loss of water due to transpiration (Robinson et al. 1998). Based on this, we assume that $NO_2$ was stressful for willow and, as a consequence, an adaptation mechanism was set into motion by decreasing their SPS, and, consequently, increasing their $R_S$. Similar results concerning the effect of air pollution stress on $R_S$ were shown by Nighat and Iqbal (2000). If the high atmospheric $NO_2$ concentration was stressful for willow, it might also be possible that the adaptation of SLA can be seen as a response to compensate the inhibition of photosynthesis caused by $NO_2$. Carreras et al. (1996) also found an increase of SLA due to stress caused by traffic-related pollution. The absence of a negative effect of $NO_2$ on $F_v/F_m$ of willow can

indicate a successful avoidance of a deleterious effect of $NO_2$ by adapting SLA and $R_S$. Normally, a healthy terrestrial plant will have a $F_v/F_m$ close to 0.83 (Papageorgiou and Govindjee 2004), while plants subjected to stress have a reduced $F_v/F_m$. Moraes et al. (2004) demonstrated a decrease of $F_v/F_m$ due to $O_3$ stress, suggesting a limited plant capacity for using photon energy, while van Hove et al. (1989) showed a positive, fertilizing, effect of ammonia on $F_v/F_m$ of poplar. In our study, we also found a higher $F_v/F_m$ for pine at monitoring stations with a higher atmospheric $NO_2$ concentration or, in other words, a lower $F_v/F_m$ at monitoring stations with a higher atmospheric $O_3$ concentration. Based on these results, together with the change of SLA for pine, it is not possible to conclude whether $NO_2$ had a positive, fertilizing effect or whether $O_3$ had a negative, toxic effect on oak and pine. Knops and Reinhart (1999) stated that nitrogen fertilization can indeed, to some point, cause an increase in SLA. No conclusion can also be made for oak, which decreased FAA with increasing atmospheric $NO_2$ concentrations, or in other words, increased FAA with increasing atmospheric $O_3$ concentrations. Normally, FAA is used as a measure of developmental instability or fitness/vitality of a tree, with a higher FAA in case of a higher fitness (Graham et al. 2003).

Generally, willow responded differently to the ambient $NO_2$ concentration compared to oak and pine: willow probably initiated an adaptive mechanism to cope with the higher atmospheric $NO_2$ concentration, while oak and pine probably benefited from the higher atmospheric $NO_2$ and lower atmospheric $O_3$ concentration. The difference in internal leaf structure can be put forward as a possible explanation of the species-dependent responses. The coefficient of palisadeness (K, %) is used as a measure of the gas exchange rate (Dineva 2004) and is related to the leaf tissue density. Giacomo et al. (2010) showed that K amounted 39% for sensitive poplar clones and 49% for tolerant poplar clones, which is also in line with the results of Dineva (2006). Based on this, willow can be seen as a more 'sensitive' species and oak as a more 'tolerant' species, since K for willow amounted 23 ± 3% and 40 ± 5% for oak. The difference in K between willow and oak is a reflection of the thicker spongy parenchyma (127 ± 28 μm) and the thinner palisade parenchyma (38 ± 9 μm) of willow, compared to oak (respectively 75 ± 12 μm and 51 ± 10 μm) and relative to the leaf thickness of willow (210 ± 38 m) and oak (151 ± 20 μm). The thicker spongy parenchyma of willow, and thus also the larger amount of intracellular spaces filled with air, can lead to a higher uptake of $NO_2$, at monitoring stations with a high atmospheric $NO_2$ concentration, than the amount that can be assimilated on time.

### 6.4.2 The response of willow, oak and pine to shade

Willow, oak and pine responded to the degree of shadow by adapting several leaf characteristics. However, the degree and nature of adaptation is different for each species, indicating the species-specific response to shade. An increase from 4% to 80% of shade caused an increase of SLA of 34%, 53% and 67% for oak, willow and pine, respectively. The adaptation of SLA to shade is discussed in Chapter 4 (see §4.4.1). Another common adaptation to low light availability is the formation of less and larger stomata, leading to a higher $R_S$ (Lichtenthaler and Babani 2004, Sarijeva et al. 2007), which was also the case for oak. Shade leaves also differ from sun leaves in their composition of photosynthetic pigments, electron carriers, chloroplast ultrastructure and photosynthetic rates (Lichtenthaler et al. 2007). Shade leaves possess shade-type chloroplasts with higher levels of chlorophyll a/b binding light-harvesting complexes (particularly those associated with photosystem II), a lower maximum photosynthetic rate, less reaction center proteins, and a higher stacking degree of thylakoids than sun leaves with their sun-type chloroplasts (Lichtenthaler et al. 2007). A low chlorophyll a/b ratio is also indicative of shade-type chloroplasts, as demonstrated for *Acer*, *Fagus*, *Tilia*, *Abies* and *Ginkgo* (Lichtenthaler et al. 2007, Sarijeva et al. 2007) and by our results, where the chlorophyll a/b ratio of oak decreased with 50% when shade increased from 4% to 80% (data not shown). The chlorophyll a/b ratio amounted 2.4 in the most shaded monitoring station ('Schoten') and 4.8 at the monitoring station the most exposed to sun light ('Ukkel'). The influence of shadow on the photosynthetic pigments of oak was also demonstrated by the 70% increase in the RCC from high to low light. However, since RCC is positively related with the total chlorophyll content on a leaf area basis and the latter decreases with increasing shade (Lichtenthaler et al. 2007), our results are rather unexpected. It is possible that the lower RCC at high light is a reflection of the natural decrease in total chlorophyll content during the summer period of, particularly, high-light plants (Lichtenthaler and Babani 2004). Increases in accessory pigments (chlorophyll b) relative to antenna pigments (chlorophyll a) in low light serve to increase the photosynthetic efficiency by enhancing photosystem II conversion of light to chemical energy (Reed et al. 2012). This in turn can cause an increase in $F_v/F_m$, since $F_v/F_m$ is a measure of the potential photosystem II efficiency of dark-adapted leaves (Eranen and Kozlov 2006). Groninger et al. (1996) also found that $F_v/F_m$ increased with shade, suggesting an increased quantum yield, and, thereby allowing more efficient energy transfer from chlorophyll to photosystem II. $F_v/F_m$ of oak increased from 0.75 under high light (4% shade) to 0.82 under low light

(80% shade). Since $F_v/F_m$ of oak under high light lies not in the optimal range of $F_v/F_m$ (0.79 - 0.85; Valladares et al. 2002), oak was probably exposed to light stress which may have caused photodamage. Photodamage is expressed as the degradation of chlorophyll, which can be another explanation of the lower RCC of oak at high light. $F_v/F_m$ of willow increased from 0.79 under high light to 0.86 under low light, which is strange for a pioneer species. One should expect a better performance of a pioneer species under high light than under low light. Also, according the relationship between CA and photosynthetic performance reported by Brewer and Smith (1995), a lower $F_v/F_m$ is expected in low light conditions, while the reverse is observed for willow in this study. The adaxial CA ranged from 76° at high light monitoring stations ('Ukkel') to 57° at low light monitoring stations ('Borgerhout'), which is line with the results of Barber et al. (2004). CA at the abaxial leaf side was not influenced by shadow. Sun leaves have thicker epicuticular wax layers than shade leaves, and, therefore, the amount of epicuticular wax rather than the wax composition is affected by the level of irradiance (Pandey and Nagar 2002).

In general, more research is necessary to find out the response of willow, oak and pine to shadow, in absence of other possible stressors, for explaining the inconsistencies found in this study. It is in any case important to use sampling locations with a similar degree of shadow and to sample leaves from unshaded positions to minimize the confounding effect of shadow when using leaf characteristics for air quality monitoring.

### 6.4.3 Active biomonitoring with willow, oak and pine

The use of Scots pine as an active biomonitor is not recommended due to high mortality, when planting pine in pots as a consequence of the high drought sensitivity of the roots. Moreover, measurements of SPS, RCC, FAA and $F_v/F_m$ on needles are very difficult and time-consuming. In contrast, willow and oak are more user-friendly for active biomonitoring studies. Willow has the advantage of reducing possible effects of genetic variability on leaf characteristics by using stem cuttings. Due to lower evapotranspiration, oak has a lower water demand than willow, and, therefore, requires less frequent refilling of the semi-automatic water supply system. In contrast with the small willow leaves, the large leaves of oak enabled the measurement of relative chlorophyll content.

## 6.5 Conclusions

This study demonstrates that the response of leaf characteristics to environmental factors is species-dependent. Shade had a strong influence on SLA, RCC and $F_v/F_m$ which overrided the effects of air pollution. As a consequence, biomonitoring must be performed at locations with a similar degree of shade and leaves need to be taken from unshaded positions. Willow and oak seem to be the most suitable species to use in active biomonitoring studies, while Scots pine is not recommended, since planting is difficult due to drought sensitivity of roots and measuring of leaf characteristics on needles is time-consuming. Willow has, in contrast with oak, the advantage of reducing possible effects of genetic variability on leaf characteristics, by using stem cuttings. SLA and $R_S$ of willow and $F_v/F_m$ of oak seem to be the most suitable leaf characteristics to gain information about the effect of ambient air quality. These leaf characteristics are easy to measure and non-destructive, which makes them particularly appropriate to use in active biomonitoring studies.

# 7

# General discussion and conclusions

In today's industrialized society, a flow of chemical compounds, such as $SO_2$, PM, $NO_2$, $CO_2$, $NH_3$ and heavy metals, is brought into the atmosphere mainly by traffic, industry, agriculture and burning of fossil fuels (see §1.1.2). All these chemical compounds can, on a short- or long-term basis, be harmful for human health, ecosystems and the quality of soil, water and air (see §1.2).

In order to protect human health and ecosystems, air quality limit values for the most common air pollutants have been established by the European Union (see §1.1.3). To quantify the concentration of air pollutants and to determine whether limit values are exceeded, traditional physico-chemical methods are used. However, long-term physico-chemical monitoring at high spatial resolution is almost impossible and very expensive. In addition, the atmospheric concentrations of $SO_2$, $NO_2$, $O_3$ and $PM_{10}$ obtained by the telemetric air monitoring networks in Belgium are condensed into one air quality index (www.irceline.be). This air quality index, which ranges from 1 (excellent air quality) to 10 (terrible air quality), is only a theoretical qualitative appreciation of the air quality, since synergistic or antagonistic interactions between air pollutants are not taken into account (Calzoni et al. 2007). A common strategy for dealing with these problems is the use of biomonitoring (see §1.3). Many researchers have used biomonitoring as a powerful cost-effective and user-friendly tool for filling the gap between the doses and responses of air pollution. Investigating the influence of one single air pollutant on plants, at (extremely) high concentrations and under

laboratory conditions, is a well-known approach in biomonitoring studies but does not provide information about the effects of actual atmospheric conditions. With this thesis, we aimed to gain more insight into the impact of ambient air quality on leaf characteristics of plants under field conditions. As the results of the present research have already been discussed extensively in the preceding chapters, the aim of the first part of this chapter is to provide an overall discussion of the findings and implications of the study. In the second part, we provide directions for further research.

## 7.1 Impact of ambient air quality on leaf characteristics of plants

### 7.1.1 Comparison between active and passive biomonitoring

Passive and active biomonitoring of air quality both have advantages and disadvantages. Passive biomonitoring has the advantage of using organisms that are already present in the ecosystem (see §1.3.1), making this approach inexpensive and time-efficient. Yet, in the present thesis, passive biomonitoring of the atmospheric $NH_3$ concentration with common oak proved that too many confounding variables mask the possible effect of $NH_3$ (Chapter 2), which brings us to the disadvantages of passive biomonitoring. The effects of variations in soil characteristics (e.g., nutrient availability, soil water limitation), and (a)biotic stressors that may have occurred in the past such as historical management (e.g., pruning intensity), soil disturbances, herbivore attacks and diseases cannot be taken into account. Genetic differences between sampled trees can lead to differences in specific leaf area (SLA), stomatal responses and fluctuating asymmetry (FA) (Pääkkönen et al. 1993, Dimitriou et al. 2006, Bonser et al. 2010). The presence of different provenances and the difficulty of taxonomic identification, due to hybridization, makes genetic pollution, and thus genetic differences in tolerance to air pollution hardly unavoidable. In addition, the used passive biomonitor should have a wide geographic distribution and fulfill a number of criteria, e.g., a comparable age and vitality.

With active biomonitoring, in contrast, organisms of the same age can be planted in the same uniform substrate and at specific sampling locations, which makes the monitoring independent of the geographic distribution of the desired biomonitor. The use of vegetatively propagated material in our studies gave us the opportunity to avoid genetic pollution. Nevertheless, the active biomonitoring with white willow to distinguish the air quality of urban and rural land use classes (Chapter 3) taught us that (i) water supply is

necessary, (ii) herbivory needs to be countered and (iii) good arrangements with land-owners need to be made so that weeding, chemical treatments etc. are done in a standardized way. To avoid water deficiency, a semi-automatic water supply system was developed for the studies in chapters 4 to 6. This system consists of a tank, which is completely filled with water and impermeable to light to avoid algae growth. The refilling frequency of the tank depends upon the amount of rain and radiation. Based on our experience, we conclude that refilling every three weeks, for willow, and every month for oak and pine is necessary during warm and dry periods. Glass fiber ropes, hanging in the water, transport the water from the tank to the potting soil via capillarity (Fig. 4.1). After one growing season, roots are growing out of the pots and taking over the role of the glass fiber ropes. To avoid snail herbivory, copper tape was attached around the pots, which successfully repelled the snails.

### 7.1.2 The species-dependent response of leaf characteristics to ambient air quality

The extent to which leaf characteristics are affected by the ambient air quality is species-specific (Larcher 2003) because of differences in tolerance, resistance and/or sensitivity of a species to air pollution stress (see §1.3.2.3). For example, when the atmospheric $NO_2$ concentration increased and the $O_3$ concentration decreased, the SLA and stomatal resistance ($R_S$) of willow increased, the SLA and maximum photochemical efficiency of photosystem II ($F_v/F_m$) of pine increased and the leaf area fluctuating asymmetry (FAA) and $F_v/F_m$ of northern red oak increased (Chapter 6). In addition, the passive biomonitoring study with common oak probably failed partly due to the fact that common oak is intermediately susceptible to short-term exposure of high atmospheric $NH_3$ concentrations (Krupa 2003) and probably also intermediately susceptible to long-term exposure of low $NH_3$ concentrations (Chapter 2). Thus, the species-dependent response complicates the species selection for biomonitoring purposes.

In general, high species sensitivity to air pollution stress is related to thinner palisade mesophyll layers and a low ratio of palisade to total (palisade and spongy) mesophyll cells (i.e., the coefficient of palisadeness) (Ferdinand et al. 2000). A thicker spongy parenchyma, and thus also a larger amount of intracellular spaces filled with air, can lead to a higher uptake of air pollutants than the amount that can be assimilated in time and, thus, lead to adaptation or avoidance strategies. Giacomo et al. (2010) showed that the coefficient of palisadeness amounted to 39% for sensitive poplar clones and to 49% for tolerant poplar clones, which is in line with the results of

Dineva (2006). In Chapter 6, we indicated that the thicker spongy and thinner palisade parenchyma of willow, compared to northern red oak, revealed that willow can be seen as a more sensitive species and oak as a more tolerant species. In principe, measuring the coefficient of palisadeness seems worthwhile to support the species selection at the start-up of a biomonitoring study. However, a reflection can be made on this statement, since defining 'sensitivity' is difficult. In §1.3.2.3, we defined 'sensitivity' as the susceptibility of an organism to environmental changes, e.g., the concentration of one or multiple air pollutants. So, the question 'Is willow more sensitive to the ambient air quality or to just one air pollutant compared to oak?' can be raised which indicates that the terms resistance, tolerance and sensitivity need to be taken with a pinch of salt. In addition, tolerance to air pollution also depends on the considered plant characteristic, as stated by Schreuder et al. (2001), which makes it possible that even the leaf characteristics of (intermediate) tolerant species, such as northern red oak, can change under increasing air pollution. In other words, it is possible that sensitive species seem unsuitable for biomonitoring air quality when only tolerant leaf characteristics are measured. Therefore, several morphological, physiological, anatomical and biochemical leaf characteristics should be measured before the suitability of a species as biomonitor can be assessed correctly.

Species selection is also based on practical considerations. Willow allows the use of stem cuttings, which (i) are easy to transport, (ii) are easy to plant, and (iii) allow to avoid genetic pollution. The fast growth of willow also has the advantage that (new) plant material can be quickly obtained. In contrast, seedlings of pine and oak are difficult to transport without damage, and the application of glass fiber ropes with these species is not a simple task due to the presence of roots and the unmanageability of large shoots. Moreover, pine roots are sensitive to drought when planted in pots, and measuring stomatal pore surface (SPS), relative chlorophyll content (RCC), FAA and $F_v/F_m$ on needles are very difficult and time-consuming.

The response of the leaf characteristics of willow (Chapter 4) as well as the practical considerations make us conclude that willow has more potential for biomonitoring ambient air quality than northern red oak and Scots pine. Gostin and Ivanescu (2007) also indicated that white willow is a good biomonitor, since its leaf phenol content and epicuticular waxes adapt to the ambient air quality. The potting conditions (e.g., occurrence of soil exhaustion) and/or harvesting in summer, however, do not allow active biomonitoring with the same willows for more than two years because of the yellowing of leaves and the occurrence of dwarf growth in the third in-leaf

season. If biomonitoring of the ambient air quality with willow is wanted for multiple years, we suggest (i) not to harvest after each sampling year to avoid exhaustion of the cutting and (ii) to use characteristics that can be measured non-destructively, such as $R_S$, SLA, malondialdehyde (MDA), polyphenols (POLY) and stable carbon isotopes ($\delta^{13}C$).

### 7.1.3 The response of morphological, anatomical, physiological and biochemical leaf characteristics of white willow to ambient air quality

Morphological, anatomical, physiological and biochemical leaf characteristics were influenced by the ambient air quality [1] in different ways, indicating the leaf characteristic-dependent responses. FAA, stomatal density (SD), drop contact angle, $F_v/F_m$, performance index, reduced ascorbate (ASC), reduced glutathione (GSH) and flavonoid content, superoxide dismutase, ascorbate peroxidase and peroxidase activity and stable nitrogen isotopes ($\delta^{15}N$) were not able to monitor the ambient air quality. Possible hypothesis about the inability of these leaf characteristics to monitor low ambient air pollutant concentrations are formulated in Chapter 4 and 5. In contrast, SLA, $R_S$, MDA, total antioxidant capacity (FRAP) and POLY content all increased and stable carbon isotopes ($\delta^{13}C$) decreased with increased PCA1-values. The response of these leaf characteristics to the ambient air quality is thoroughly discussed below.

In Chapter 4, the increase of **SLA** with an increase of PCA1-values was explained as follows: **(i)** white willow decreased its SLA to minimize the uptake of pollutants (Wen et al. 2004) by decreasing leaf area, increasing leaf density and/or thickness (Tiwari et al. 2006) and/or increasing leaf starch concentration (Schmitt et al. 1999) under high atmospheric $\mathbf{O_3}$ concentrations or **(ii)** white willow increased SLA due to compensatory growth to reduce the inhibition of photosynthesis (Canas et al. 1997), caused by a high atmospheric $\mathbf{NO_2}$ concentration. In addition, since Knops and Reinhart (1999) stated that nitrogen fertilization can positively influence growth and increase SLA, we could not ignore the possibility of a **fertilization** effect of atmospheric $NO_2$ on SLA. We also demonstrated that $\mathbf{R}_S$ was positively correlated with PCA1, which means that an increase in atmospheric $NO_2$ concentration led to an increase in $R_S$, or that an increase in atmospheric $O_3$ concentration led to a decrease in $R_S$ after two years of exposure. The

[1] Ambient air quality was described by a site-specific value (PCA1), which related positively with the mean atmospheric $NO_x$ and negatively with the mean $O_3$ concentrations over the in-leaf season (Chapter 4)

increase in $R_S$ due to an increase in **$NO_2$** concentration indicates a toxic effect of $NO_2$, **ruling out the possibility of a fertilization effect of $NO_2$**, and can be seen as an adaptation to minimize the uptake of atmospheric $NO_2$, while optimizing $CO_2$ uptake and reducing the loss of water due to transpiration (Gostin 2009). In addition, the atmospheric $O_3$ concentration could also be toxic, leading to a decrease in $R_S$. $O_3$ is known for strengthening stomatal patchiness, i.e., the heterogeneous aperture of stomata on the leaf surface, and for causing sluggish stomatal response (Paoletti and Grulke 2005 and references herein). The mechanism of sluggish stomatal behavior is still largely uncomprehended, but it is known that it leads to incomplete stomatal closure. The loss of stomatal control, due to air pollution, was also found by Atkinson et al. (1991); Reiling and Davison (1995) even reported that stomata did not completely close anymore at night as a result of air pollution. Moreover, Maier-Maercker (1989) found that guard cell walls of *Picea abies* delignified after $O_3$ exposure, resulting both in greater stomatal apertures, because of a reduction of the mechanical resistance towards guard cells, and in a slower water release from guard cells, as cellulose has a higher affinity for water than lignin (Paoletti and Grulke 2005 and references herein). At first sight, the change in $R_S$ was achieved by a change in SPS and SD due to PCA1, but the so-called adaptation of SD due to air pollution was attributed to the extent of cell expansion instead of stomatal differentiation (Chapter 4). The share of SPS in determining $R_S$ was also shown by the active biomonitoring with white willow in urban - more polluted - areas versus rural areas (Chapter 3).

In Chapter 5, we showed that the leaf **MDA** content of willow was higher at locations with a high mean atmospheric $NO_2$ and low mean atmospheric $O_3$ concentration than at locations with a high $O_3$ and low $NO_2$ concentration. This indicates that the high atmospheric $NO_2$ concentrations caused **oxidative stress**. The oxidative stress lead to the production of reactive oxygen species (ROS), which led to the peroxidation of poly-unsaturated fatty acids, and, thus, the enhanced production of MDA. Consequently, a toxic effect of high atmopsheric $O_3$ concentrations can be ruled out, since a high $O_3$ concentration would lead to an increased MDA content, which was not the case in this study. A defense mechanism against the oxidative stress was activated by increasing the total **POLY content**, which is also visible in Fig. 5.1 (Chapter 5), and, thus, also by increasing the **FRAP**.

The occurrence of oxidative stress at monitoring stations with a high atmospheric $NO_2$ concentration and low $O_3$ concentration, indicates that the atmospheric $NO_2$ is toxic and, thus, we can say that the adaptation of SLA and $R_S$ has been established due to high atmospheric $NO_2$ concentrations

rather than high $O_3$ concentrations. The lower $\delta^{13}\mathbf{C}$, due to changes in stomatal conductance and/or biochemical characteristics (e.g., chlorophyll degradation) that negatively affect photosynthesis (Farquhar et al. 1982, Dawson et al. 2002), also indicated a toxic effect of atmospheric $NO_2$. To obtain an integrated picture of the influence of atmospheric $NO_2$ on the leaf characteristics of willow, a schematic overview was made (Fig. 7.1).

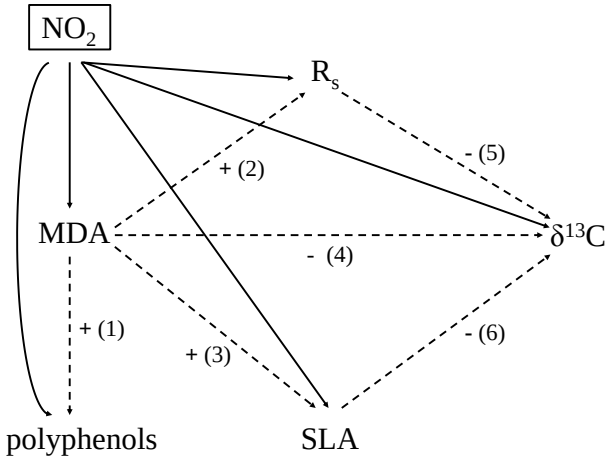


Figure 7.1: Indicative schematic overview of the influence of atmospheric $NO_2$ concentrations on leaf characteristics and the relationships (positive +, negative -) between these characteristics of white willow. Full lines indicate direct influences; dashed lines indicate indirect influences, numbers are used in the explanation

High atmospheric $NO_2$ concentration can negatively influence all the leaf characteristics mentioned in Fig. 7.1 directly and/or indirectly. The increased production of ROS due to high atmospheric $NO_2$ concentrations, which leads to lipid peroxidation, can trigger the antioxidant system by increasing the production of polyphenols (1, Fig. 7.1). In turn, leaf MDA content can enhance the production of free radicals (e.g., $H_2O_2$), which will react with proteins, DNA and membrane lipids and eventually lead to reduced photosynthesis (Sharma and Davis 1997). This reduced photosynthesis can be minimized by compensatory growth, leading to an increased SLA (3, Fig. 7.1). Furthermore, $H_2O_2$ has the ability to act as an effector of stomatal closure (2, Fig. 7.1) by activating $Ca^{2+}$ channels and modulating the $Ca^{2+}$ cytosol concentration (Pei et al. 2000, Desikan et al. 2004). The

biochemical limitations (4, Fig. 7.1), as well as stomatal (5, Fig. 7.1) and morphological limitations (6, Fig. 7.1), can decrease the photosynthetic performance (Richardson et al. 1990), and, therefore, also $\delta^{13}C$. It must be noted that the explanation of the schematic overview (Fig. 7.1) is indicative and needs to be supported by further studies.

### 7.1.4 Drawbacks of biomonitoring studies

Biomonitoring with leaf characteristics of white willow seemed to be limited to obtain information about the effects of atmospheric $NO_2$ concentrations. If, for example, information about $O_3$ is needed, we suggest to use the sensitive species *Nicotiana tabaccum*, while lichens or mosses are applicable for measuring effects of atmospheric $NH_3$ and $SO_2$ concentrations. Furthermore, biomonitoring (with white willow) does not allow to distinguish between the effects of different air pollutants nor to investigate the nature of the interactions between air pollutants, which can be antagonistic (Jäger et al. 1992) or synergistic (Tiwari et al. 2006). We can assume that some interaction effects between the different pollutants occurred, since the atmospheric $NO_2$ concentration could not explain all the observed between-site variability in leaf characteristics.

In addition, biomonitoring with white willow does not allow to quantify actual concentrations of atmopsheric $NO_2$ nor provides high temporal resolution data, i.e., on a monthly, daily or even hourly basis as can be obtained by physico-chemical methods. This emphasizes that the final goal of biomonitoring is not to replace the traditional physico-chemical approach, but that both methods provide complementary data. Using a site-specific value for the ambient air quality (PCA1) has the drawback of not taking into account the high monthly, daily and hourly variation of air pollutant concentrations, since all concentrations are averaged over the exposure period. This means that no account is taken of variations in peak concentrations between the sampling locations, occurring before noon or at optimal meteorological conditions when stomata are usually fully open. Biomonitoring ambient air quality under field conditions is also complicated due to the presence of a plethora of (abiotic and biotic) successive and/or simultaneous factors, influencing the response of leaf characteristics (see §1.3.2.3).

### Shade

Under shaded conditions, the C uptake per unit leaf biomass is lower than under full light conditions (Van Hees and Clerkx 2003). To maintain a positive C balance, a plant can alter its biomass partitioning and make physiological, morphological, anatomical and/or biochemical adjustments (Van Hees and Clerkx, 2003). Since white willow is a shade-intolerant species, adaptation of several leaf characteristics to shadow was inevitable in our study. Shade leaves were (i) thinner than sun leaves, due to the reduction of the palisade parenchyma thickness and (ii) more wettable due to the reduced biosynthesis of cuticular waxes (Chapter 4). The higher wettability of the abaxial leaf surface compared to the adaxial surface also partly indicated the influence of shade. In addition, shade leaves had a lower level of leaf ASC and GSH (Chapter 5). This may be due to light-dependent changes in rates of GSH breakdown or export, a restricted conversion of $\gamma$-glutamylcysteine to GSH, a decreased availability of glycine and a down-regulation of GDP-D-mannose pyrophosphorylase, L-galactose 1-P phosphatase and L-galactono-1,4-lactone dehydrogenase (Logan et al. 1996, Noctor et al. 1997, Massot et al. 2012).

Shade leaves also had a higher $F_v/F_m$ and performance index (Chapter 4). The better performance of willow under shaded conditions is, however, strange for a pioneer species: a pioneer species is expected to perform better under high light than under low light. On the one hand, it is possible that the quantum yield increased in shade-grown plants, allowing a more efficient energy transfer from light-harvesting chlorophyll to photosystem (PS) II instead of PS I (Demmig and Bjorkman 1987, Groninger et al. 1996, Eranen and Kozlov 2006). On the other hand, it is possible that the higher $R_S$ of leaves under shaded conditions caused a reduced uptake of atmospheric $NO_2$, leading to a better performance (less air pollution stress) in shaded habitats compared to sunny habitats. The formation of less and larger stomata, leading to a higher $R_S$ (Lichtenthaler and Babani 2004, Sarijeva et al. 2007), is a common adaptation to low light availability.

To conclude, the effect of shade needs to be taken into account by choosing sample sites with a similar degree of shade, by taking leaves from unshaded positions and/or by measuring leaf characteristics that are less sensitive to shade, such as leaf MDA, POLY content and $\delta^{13}C$. If it is not possible to use sample sites with a similar degree of shade, one needs to take hemispherical photographs (Chapter 4) and add shade as a possible explanatory variable to the statistical model. If shade seems to affect the measured plant characteristics, reducing this effect can be done by removing data of highly

shaded sample sites (Chapter 4). However, the influence of shade cannot be accounted for completely, due to unknown interaction effects with other environmental conditions.

### Inter-tree and intra-shoot variability

Each plant and each leaf of a plant has its own tolerance against air pollution stress (Niinemets 2010), which leads to a high inter-tree variability (i.e., variability between stem cuttings at the same monitoring station) and intra-shoot variability (i.e., variability between shoots of the same stem cutting) of the response of leaf characteristics of willow. In addition, the variability in the microclimate, i.e., the climate a specific plant/leaf is exposed to, and plant-specific factors such as age, stage of development and position of the leaf on the plant can also lead to inter-tree and intra-shoot variability of leaf characteristics (Cowart and Graham 1999, Gunn et al. 1999, Poorter et al. 2009). For example, leaves produced under higher air temperatures have a lower SD compared to leaves produced under lower temperatures (Beerling and Chaloner 1993), and SPS is controlled by phytohormones, such as abscisic acid, cytokines and gibberellins which depend on the development stage of the plant (Larcher 2003). This - naturally occurring - variability complicates a biomonitoring study on responses of leaf characteristics to ambient air pollution; Loponen et al. (1998) found a high inter-tree variability in phenol content, which made it difficult to find consistent differences between leaf phenol content in trees in polluted versus control areas. More research is needed to investigate the possible factors that lead to the high inter-tree and intra-shoot variability of almost all studied morphological, anatomical, physiological and biochemical leaf characteristics of willow (Chapter 4 - 5). In any case, a large sample size need to be used in future research in order to take the inter-tree and intra-shoot variability as much as possible into account.

## 7.2 Suggestions for further research

This thesis has contributed to the current knowledge of biomonitoring ambient air quality with leaf characteristics of trees and has formulated some important recommendations on the use of active biomonitoring. Nevertheless, there remain issues that could be addressed in future research, in addition to the research suggestions mentioned in the previous chapters.

Firstly, leaf characteristics (SLA, $R_S$, MDA, POLY and $\delta^{13}C$) of willow proved to adapt to the ambient $NO_2$ concentration, which makes them potentially useful to monitor traffic emissions. However, a large part of the between-site variability and the presence of the large inter-tree and intra-shoot variability are still unexplained. Therefore, we suggest (i) to identify the factors that lead to the high inter-tree and intra-shoot variability, j(ii) to separate the air pollution effect (signal) from variation caused by other factors (noise) and (iii) to investigate and understand the contribution of antagonistic and/or synergistic interactions of atmospheric $O_3$, $SO_2$ and $PM_{10}$ concentrations on the described response of leaf characteristics to atmospheric $NO_2$ concentrations. The last two points can be (partly) investigated by fumigation experiments in which ambient concentrations of $NO_2$, $O_3$, $SO_2$, $PM_{10}$ are combined with meteorological conditions. In addition, a fumigation experiment can also be used to investigate the atmospheric $NO_2$ concentration from which a signal in SLA, $R_S$, $\delta^{13}C$ and leaf MDA and POLY content can be recorded, so that the detection limit can be determined.

Secondly, biomonitoring the ambient air quality with white willow is not possible, since only information about the ambient $NO_2$ concentration is obtained. It would be interesting to evaluate (i) whether other leaf characteristics of willow, not included in the thesis, can provide information about atmospheric $O_3$, $SO_2$ and $PM_{10}$ concentrations, (ii) whether other plant species can be used to biomonitor the ambient air quality and (iii) whether northern red oak can be used to monitor the atmospheric $O_3$ concentration. The latter can be investigated by means of biochemical characteristics (e.g., MDA). In addition, the biomonitoring studies in this thesis are based on the impact of atmospheric $NO_2$ concentrations during several months. It would be interesting to find a leaf characteristic (of white willow) that obtains information about the impact of atmospheric $NO_2$ concentrations with a finer temporal resolution (e.g., several weeks).

Thirdly, human biomonitoring is emerging by using blood, hair and urine as biological markers (Hohenblum et al. 2012). Investigating the response of

ambient $NO_2$ concentrations on the MDA content in humans living in urban and rural areas would be an interesting research topic. Lipid peroxidation also occurs in humans and involves the oxidative deterioration of polyunsaturated fatty acids in biomembranes, generating a variety of aldehydic products, including MDA (Karatas et al. 2002). If a relationship between the MDA content in humans and willow could be established, the ability to use plants for obtaining information about human health is near. In addition, a standardized protocol for measuring MDA and phenol content of willow leaves needs to be developed to reduce the measurement error.

# 8
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