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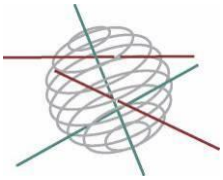

Agro-food

FINAL REPORT

IMPACT OF TROPOSPHERIC OZONE ON FOOD AND FEED QUALITY OF BRASSICA SPECIES

"OFFQ"

SD/AF/02

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Acronyms, abbreviations and units

APPO	Association pour la Promotion des Protéagineux et des Oléagineux
APX	Ascorbate peroxidase
A_{sat}	Light saturated CO_2 assimilation (µmol CO_2 m^{-2} s^{-1})
ASC	Ascorbate
AOT40	Accumulated Ozone exposure over a Threshold of 40 ppb during daylight hours (ppm.h)
CUO	Accumulated ozone uptake (mmol m^{-2})
DAE	Days after emergence
DHA	Dehydroascorbate
DHAR	Dehyrdoascorbate reductase
DW	Dry weight
F_m	Maximum fluorescence
F_v	Variable fluorescence
FW	Fresh weight
GDP	Guanosine diphosphate
g_s	Stomatal conductance (mol H_2O m^{-2} s^{-1})
g_{stO3}	Stomatal conductance to O_3 (mol O_3 m^{-2} s^{-1})
GSH	Glutathione
GSL	Glucosinolate
GR	Glutathione reductase
LAI	Leaf area index (m^2 leaf/m^2 ground area)
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
MLA	Maximum leaf area (m^2 leaf/m^2 ground area)
NADPH	Nicotinamide adenine dinucleotide phosphate
NF	Unfiltered OTC
NF^+	Unfiltered OTC + 20 ppb O_3
NF^{++}	Unfiltered OTC + 40 ppb O_3
NIR	Near Infra Red
OP	Open field plot
OTC	OpenTop Chamber
O_3	Ozone
PAR	Photosynthetic Active Radiation (µmol m-2 s-1)
PCD	Programmed cell death
PI	Vitality Performance Index
PLA	Plant leaf area (m^2)
POD_x	Phytotoxic ozone dose above a threshold of x nmol O_3 m^{-2} s^{-1}
POX	(unspecific) Peroxidase
PS I & II	Photosystem I & II
R1000SW	Relative 1000 seed weight
RFMW	Relative fresh marketable yield
RTDW	Relative total dry weight
ROS	Reactive oxygen species
ROY	Relative oil yield
RO%	Relative oil percentage
RSY	Relative seed yield
S1	Sampling point at the vegetative stage
S2	Sampling point at the generative stage
SOD	Superoxide dismutase

TAC	Total (water soluble) antioxidative capacity
rASC	Reduced ascorbate
tASC	Total ascorbate
rGSH	Reduced glutathione
tGSH	Total glutathione
TOC	Tocopherol
TT	Thermal time above 0°C (° days)
UA	University Antwerp
VAR	Veterinary and Agrochemical Research Centre (partner 1)
VPD	Vapour pressure deficit (kPa)
Φ_{PSII}	Actual quantum yield of photosystem II

SUMMARY

Context

Human activities are having an unprecedented impact on the global environment and major climatic changes have been predicted (and are already being observed) as a consequence of this. Ozone (O_3) is a naturally occurring chemical present in both the stratosphere (the 'ozone layer', 10 – 40 km above the earth) and in the troposphere (0 – 10 km above the earth). Whereas stratospheric O_3 protects the Earth's surface from solar UV radiation, tropospheric O_3 is (after CO_2 and CH_4) the third most important greenhouse gas (Denman *et a*l, 2007; Solomon *et al*, 2007). Besides its role as a direct greenhouse gas, O_3 has been identified as the most important rural air pollutant, affecting human health and materials, as well as vegetation (WGE, 2004).

Increased emissions associated with fossil fuel and biomass burning (Gauss *et a*l, 2006; Denman *et al*, 2007), long-distance and even intercontinental transport of O_3 precursors have resulted in a steady increase in O_3 concentration in rural areas hundreds and thousands of kilometres from the original sources of pollution (Prather *et al*, 2003). Nearly one-quarter of the Earth's surface is currently at risk from mean tropospheric O_3 in excess of 60 ppb during midsummer with even higher local concentrations occurring (Fowler *et al*, 1999 a,b). This is well above the mean concentration of 40 ppb that has been determined for damage to sensitive plant species (Fuhrer *et al*, 1997; Mills *et al*, 2000; LRTAP Convention, 2007). Several scenarios indicate that concentrations of tropospheric O_3 might further increase throughout the 21st century (Gauss *et al*, 2003); simulations for the period 2015 through 2050 project increases in tropospheric O_3 of 20 to 25% (Meehl *et al*, 2007). The global patterns of exposure of vegetation to O_3 are also changing. A prediction of the differences in annual global mean surface O_3 concentrations from 1990 to 2020 has recently been modelled by Dentener *et al* (2005), showing increases in all major agricultural areas of the northern hemisphere with large spatial variation. In North America and Western Europe reductions in peak O_3 concentrations are expected (e.g. Gardner & Dorling, 2000) but these changes are offset by the predicted increases in global background tropospheric concentrations (NEGTAP, 2001). Furthermore, in parts of Asia, Latin America and Africa, these increases in background concentrations are combined with trends of increased emissions of O_3 precursors, suggesting that current and future impacts of O_3 on crops and forests in these areas will be very significant (Emberson *et al*, 2001). Other important interactions may arise from the fact that O_3 as such alters the performance of herbivorous insect pests and of plant pathogens, which will themselves be influenced by climate change, e.g. as a result of greater survival under milder winter conditions.

Many studies have been conducted on the impacts of O_3 pollution on vegetation, ranging from effects at the cellular level to predicting impacts on a regional and international scale (e.g. EPA, 1996). O_3 damage to plant tissues includes visible leaf injury, decreased photosynthesis and increased senescence, which has significant repercussions for the yield and quality of major agricultural crops, biodiversity and forest health. These effects are primarily induced by an increased production of reactive oxygen species (ROS), both outside and inside the plant cell, which is a common feature of biotic (pathogens, insects) and edaphic stresses (drought, high light, UV, cold…) in plants. These stress conditions may activate the same, or at least overlapping, signal transduction pathways involving salicylic and jasmonic acid and ethylene. Consequently, O_3 itself can modify the response of plants to a

range of naturally occurring environmental stresses such as drought (Bell, 1987). This explains why O_3 has been recognized as a good tool to study signalling cascades that involve apoplastic ROS formation in the regulation of gene expression and can be used to improve our understanding of the complex network of interacting signalling pathways involved in plant defence mechanisms (Rao *et al*, 2000).
The antioxidant defence response influences the production of secondary metabolites such as vitamins and natural toxins e.g. glucosinolates (GSLs). The production and breakdown of GSLs is an important inducible defence system that is found exclusively in plants of the family *Brassicaceae*. Their breakdown products have been shown to possess a range of antifungal, antibacterial and antimicrobial activities (Fenwich *et al*, 1989). Most importantly, these biochemicals have been attributed anticarcinogenic properties and therefore considerable effort is being put in trying to understand and manipulate metabolic pathways leading to an increase of these anti-carcinogenic compounds in the human diet. On the other hand, GSLs exert anti-nutritional and even toxic effects, especially in animal feedstuffs, such as rapeseed meal, decreasing the digestibility and causing e.g. goitre and haemolytic anaemia. From a nutritional point of view, vitamin C (ascorbic acid, ASC) and E (α-tocopherol, TOC) are antioxidants with mainly beneficial health effects. The evidence is accumulating that diets rich in plant antioxidants derived from fruits and vegetables are associated with lower risks of coronary heart disease and cancer.
The close phylogenetic relationship of *Brassica* crops with the model plant species *Arabidopsis thaliana*, for which the entire genome sequence has been available since 2000, provides another important argument for adopting *Brassica* as the paradigm for transfer and testing of fundamental knowledge to crop plants.
In conclusion, there is no doubt that predicted increases in tropospheric O_3 will impact on future agro-ecosystems and their management. This study aims to contribute to the risk assessment of the impact of these predicted increases in tropospheric O_3 on the yield, quality and safety of *Brassica* species as primary source for human nutrition and animal feed. The influence of elevated O_3 on secondary metabolism such as antioxidants and GSLs will also improve our understanding of plant defence responses and signaling pathways in general. Increasing knowledge of the plant-environment interactions will surely provide novel strategies to stabilize agricultural yield and quality in a fluctuating environment. This knowledge is imperative to be able to detect, monitor and understand the full impact of our changing environment, in order to identify the risks and justify the appropriate actions (EPSO, 2005).

Objectives

- To contribute to the risk assessment of increasing tropospheric O_3 pollution by the establishment of reliable O_3 dose/exposure response relationships for oilseed rape and broccoli crops. Therefore not only the economic yield is considered, but also quality traits of the marketable end products are taken into account. This will be achieved by exposure of oilseed rape and broccoli to different O_3 levels under "near-field" conditions and analysis of the consumable end product.
- To develop a flux model for the estimation of the O_3 uptake by oilseed rape and broccoli as influenced by climatic parameters such as air humidity, solar radiation, temperature and crop phenology. This will be achieved by relating changes in the plants' stomatal conductance, obtained through field measurements, to hourly means of these climatic parameters, evolution of crop growth and O_3 concentrations.

- To determine the impact of increasing tropospheric O_3 concentrations on changes in secondary metabolism of *Brassica* species, especially on the antioxidant (vit C & E, glutathione (GSH)) and GSL composition because these compounds are highly important in relation to the health and safety aspects of human food and animal feed.
- It will be further investigated whether the O_3 induced changes in glucosinolate content and composition of the marketable end products may have consequences on the human diet and animal feed intake.
- To identify physiological and biochemical biomarkers for O_3 stress by investigating the interaction between stress induction, changes in secondary metabolites and yield effects. Therefore the physiological assessments of plant stress responses (photosynthesis and chlorophyll fluorescence) will be linked to biochemical analyses of antioxidants and GSLs at the leaf level.
- Elucidation of the impact of a long term (not acute) increase in tropospheric O_3 levels on leaf/plant metabolism and defence pathways by means of transcriptome analysis.

Conclusions

The impact of increasing tropospheric O_3 concentrations on the food and feed safety and security of *Brassica* species proved to be dependent on the nature of the marketable end product. This was clearly illustrated by the species presented in this study. For oilseed rape (*Brassica napus*) the seed production represents the economic value of the crop, whereas for broccoli (*Brassica napus* cv italic), it is the fresh vegetable, harvested before seed set, that will appear on the market.

Our primary aim was to develop quantitative O_3 dose-response relationships for risk assessment of present and future O_3 damage to these *Brassica* species. The functions were not only based on ground level O_3 concentrations but also on the biologically more relevant O_3 uptake. Therefore the variation of the stomatal O_3 uptake was modelled as a function of climatic conditions (soil moisture, air humidity, temperature, global radiation) and phenology. For the subsequent flux modelling two approaches were explored. The first model is an empirical multiplicative model which is currently used for flux-based O_3 risk assessment for vegetation over Europe. The second model, referred to as the coupled model, is a more mechanistic model. As the comparison of the modelled and measured stomatal conductance did not indicate a clear distinction between the predictive performance of both models, only the empirical model was used for further calculation of the absorbed O_3 dose and comparison of concentration versus dose based O_3-yield responses.

In comparison to the current situation, seed yield losses of spring oilseed rape may be reduced by 30% within 100 years if future ambient 7 or 12 hr average O_3 concentrations increase to a range of 51 – 75 ppb, as predicted by Assessment Report Four (Meehl *et al*, 2007). Oil yield will be even more affected due to an additional decrease of the oil percentage. Since oilseed rape is the third most important world source of vegetable oil, this implicates a considerable additional economic loss for farmers. Such an effect should also be taken into account for the estimation of biofuel production under future scenarios of increasing tropospheric O_3 levels. There was also a shift in the fatty acid composition of the vegetable oil derived from seeds of oilseed rape. Oleic acid (18:1), which constitutes about 60% of the total fatty acid content, declined significantly in favour of linoleic acid (18:2). The suppression of monounsaturated fatty acids coincided with a positive response of the % saturated fatty acids. After removal of the oil, the residual rapeseed meal contains proteins that are used as a feed supplement. In general, there is an

inverse relationship between seed oil and protein content and also in our study as the seed protein content was significantly increased by O_3 exposure. In rapeseed oil, vitamin E occurs as a mixture of α- and γ- tocopherol (TOC). The observed decrease in vitamin E content was due to a reduction of γ-TOC which may have an effect on the oxidative stability and storage life of rapeseed oil. α-TOC, the most active form of vitamin E in humans, was not influenced by O_3. Ozone exposure did not result in any significant changes in the GSL content or composition of *Brassica napus* seeds so no consequences are to be expected with regard to feed safety. However, it must be mentioned that the GSL level of the investigated spring cultivar (Ability), was very low and consequently statistically significant changes are more difficult to detect. Therefore it would be interesting to investigate this in e.g. winter cultivars of *Brassica napus* with a higher endogenous GSL content.

Despite the fact that elevated O_3 exposure did not have an effect on the fresh weight of broccoli vegetables, the quality was undoubtedly influenced. Aliphatic GSLs (glucoiberin and glucoraphanin) showed a strong tendency to increase as a consequence of higher O_3 uptake during crop growth although this did not result in a general increase of the total content since the indol fraction (mainly glucobrassicin and neoglucobrassicin) was significantly decreased. With a season long increase of the ground-level O_3 concentration by 40 ppb during daylight hours, the ratio of aliphatic/indol GSLs in broccoli vegetables was significantly raised from 0.97 to 1.72 which may be considered beneficial with regard to their anticarcinogenic properties. Other positive consequences of more elevated O_3 exposure on broccoli quality were an increase of the protein concentration and of the antioxidant GSH.

The effects of elevated O_3 exposure on yield quantity were in accordance changes in canopy development and physiological performance of the upper canopy leaves of both plant species. The reduction of the seed yield *of Brassica napus* cv Ability can be caused by a more rapid decrease in the photosynthetic performance of the leaves due to earlier senescence. The absence of any biomass or yield effects for broccoli may be due to the fact that the CO_2 assimilation in the upper canopy leaves was not yet affected by O_3 at the time of harvest. This may be due to the short growth period and limited O_3 exposure but possibly the detrimental O_3 effects on marketable yield are not yet measurable because broccoli harvest occurs before senescence sets in.

Most consequences of long term chronic O_3 exposure in plants appear at a late growth stage, and this was also the case for changes in leaf antioxidants. However, here as well both species responded differently. In general, in oilseed rape there was an increase of the antioxidant level, especially of the water soluble antioxidants, whereas in broccoli leaves TOC levels were reduced in response to O_3. As in the vegetables, the ratio aliphatic/indol GSLs in broccoli leaves showed a tendency to increase under elevated O_3 (not significant). Microarray results showed a high variability in gene expression of field grown oilseed rape and broccoli. In conjunction with the low O_3 exposure, this may explain why no clear shifts in metabolic pathways were detetected. An up regulation of PS I genes in the microarray could not be confimed through RT-PCR analysis. In contrast, effects on enzymatic activities and metabolites involved in antioxidative defense was clearly detected and were most pronounced during generative growth in oilseed rape. In general, enzymes in direct contact with ROS showed an increased activity, especially APX, which is activated post transcriptionally. An increased pool of ASC and GSH is available in ozone exposed oilseed rape plants during generative growth. A higher redox potential for ASC is maintained by an increase in MDHAR activity, by an upregulation of the corresponding gene.

Our findings clearly illustrate that O_3 not only has an influence on primary (e.g. CO_2 assimilation) but also on secondary metabolism and antioxidative defence pathway in these plants which has repercussions for both quantity and quality of crop yield. For *Brassica napus* the yield losses associated

with increasing tropospheric O_3 concentrations are of economic importance, but a distinction must be made between the loss in oil production and increase in protein concentrations. Changes in fatty acid composition may have and influence on the nutritional quality of rapeseed oil, whereas the reduction in γ-TOC may have consequences for its oxidative stability and storage life. Although no yield reduction was observed for fresh broccoli vegetables, O_3 did have a significant effect on their quality. Especially the increase in the ratio of aliphatic/indol GSLs may be important with regard to the anticarcinogenic properties for which broccoli consumption is highly recommended.

Contribution of the project in a context of scientific support to a sustainable development policy

Ozone has long been recognised as causing losses in crop productivity and changes in the quality of agricultural products. There is now a strong demand from policy makers for the quantification of O_3 damages to be fed into cost-benefit analysis of emission control strategies (Holland *et al*, 2006). This project supplied such information for some major Brassica crops: *Brassica napus* (oilseed rape) and *Brassica oleracea* (broccoli).

Economic losses are expected for oil seed rape if O_3 concentrations continue to rise. In comparison to the current situation, seed yield losses of spring oilseed rape may be reduced by 30% within 100 years if future ambient 7 or 12 hr average O_3 concentrations increase to a range of 51 – 75 ppb, as predicted by Assessment Report Four (IPCC, Meehl *et al*, 2007). Oil yield is even more affected due to an additional decrease of the oil percentage which will need to be taken into account for the estimation of biofuel production under future scenarios of increasing tropospheric O_3 levels. Based on our data the critical AOT40 (Accumulated Ozone exposure over a threshold of 40 ppb) to prevent 5% seed or oil yield are respectively 3.7 and 3.2 ppm h from emergence until harvest, which implies that the presently accepted critical level of 3 ppm h for agricultural crops (UNECE) will also protect spring oilseed rape.

For the quantification of O_3 responses the flux-based method is preferred on the grounds that it estimates yield losses and quality effects against received dose of O_3, rather than against simple exposure to ambient levels. However, so far the flux-based method could only be applied to wheat and potato. We now developed such a model for spring oilseed rape and broccoli so that a wider range of crops may be included in the O_3 risk assessment. The flux-based critical level above which 5% yield reduction for oilseed rape may be expected, is estimated at a POD_6 (Phytotoxic Ozone dose above a threshold of 6 nmol s^{-1} m^{-2} projected leaf area) of 4.4 mmol m^{-2} (for seed production) and 3.9 mmol m^{-2}. PLA (for oil production).

These concentration- and flux-based critical levels can be compared to modelled O_3 concentrations and fluxes for 50 km x 50 km grid squares across Europe as supplied by EMEP (European Monitoring and Evaluation Programme*)* to identify those areas that are most at risk for O_3 damage to oilseed rape, at present but also as predicted for the future.

This project also illustrates that the focus on yield changes could however result in a misleading risk assessment and economic extrapolations since also qualitative attributes of the harvested products may be affected by O_3. Depending on nature of these quality traits for industrial processing and consumer's health, the consequences of increasing tropospheric O_3 concentrations may have beneficial or detrimental consequences on the food and feed chain.

Keywords

Tropospheric O_3, risk assessment, *Brassica napus*, *Brassica oleracea*, oilseed rape, broccoli, yield, quality, dose response, antioxidants, glucosinolates, vitamins, food safety & security

1 INTRODUCTION

Tropospheric (0 – 10 km above the earth) ozone (O_3) is the third most important greenhouse gas and its concentration is still increasing. Simulations for the period 2015 through 2050 predict increases in tropospheric O_3 of up to 25% (Meehl *et al*, 2007). Besides its role as a direct greenhouse gas, O_3 has been identified as one of the major phytotoxic air pollutants and represents an important risk for natural vegetation, forests, crops and grasslands (Fuhrer et al, 1997; Pleijel et al, 2004, Mills et al, 2011). O_3 damage to plant tissues includes visible leaf injury, decreased photosynthesis and increased senescence, which has significant repercussions for the yield and quality of major agricultural crops, biodiversity and forest health. There is no doubt that predicted increases in tropospheric O_3 will have an impact on future agro-ecosystems and their management. At present however, the major current projections of global food production under atmospheric change scenarios do not account for the damaging effect of rising O_3 and current risk assessment tools do not sufficiently consider its interaction with other climatic changes (Ashmore and Bell, 1991; Long et al, 2005; Easterling *et al*, 2007). For the development of regional emission reduction strategies and related economic cost-benefit analyses, it is absolutely necessary to determine reliable **ozone dose-response relationships** that quantify the consequences of O_3 pollution on vegetation as part of the integrated risk assessment.

Because of their importance to the world economy, health (*Brassica* vegetables contain vitamins and anti-carcinogenic compounds; rapeseed oil contains an excellent balance of polyunsaturated fatty acids) and the environment (rapeseed oil can serve as a biofuel or a renewable resource for industrial applications) the objective of the present research was to investigate the impact of a further increase tropospheric O_3 concentrations on two *Brassica* species: spring oilseed rape (*Brassica napus* L.) and broccoli (*Brassica oleracea* L. cv. Italica).

Ozone has proven to decrease food quantity and change its quality. From an agronomic point of view, yield and nutritional quality are of utmost importance – with the former aspect being more extensively studied in the past than the latter (Ashmore, 2005). This focus on yield could however result in a misleading risk assessment and economic extrapolations, especially in those cases where the qualitative attributes of the harvested product are crucial for industrial processing and consumer's health. Crop quality may be affected either by changes in primary metabolite production and/or assimilate allocation and transport (carbohydrates, proteins,…) but also by changes in secondary metabolism. In *Brassica* species, the phytochemicals arising from these pathways include not only powerful antioxidants such as **vitamin C (ascorbic acid, ASC) and E (α-tocopherol, α-TOC)**, but also **glucosinolates (GSLs)**. These compounds possess a wide range of antifungal, antibacterial and antimicrobial activities and have been attributed anti-carcinogenic properties (Talalay and Fahey, 2001). In animal feed however, glucosinolates decrease digestibility and may cause goitre and haemolytic anaemia if supplemented at excessive rates (Stoewsand, 1995). O_3 induced changes in food and feed quality have been studied in only a limited number of crops and most investigations deal with carbohydrate and crude protein content. Despite numerous studies on the biochemical and molecular mechanisms of oxidative stress, at present only very little information exists on shifts of secondary metabolites in marketable yield products (grains, tubers, fruits, vegetables). Changes in plant chemistry and leaf structure may also influence plant-pathogen interactions (Tiedemann and Firshing, 1993; Plessl et al, 2007; Bidart-Bouzat and Imeh-Nathaniel, 2008; Himanen et al, 2009). Cultivated *Brassica* species

are grown in many countries and include important oil, vegetable and condiment crops. However, considerable improvement in yield, quality, robustness, range and input efficiencies is required if the full potential benefits of *Brassica* crops are to be realized. Due to their close phylogenetic relationship with the important model species *Arabidopsis thaliana*, for which the entire genome sequence has been available since 2000, it was anticipated that knowledge transfer for *Brassica* crop improvement would be straightforward. However, although the physiology and developmental biology of *Arabidopsis* and *Brassica* are very similar, the genomes of Brassica species are very much more complex than that of *A. thaliana*, as a result of multiple rounds of polyploidy during their ancestry. A more thorough investigation of **O_3 induced changes in metabolic pathways through an integrated combination of physiological, biochemical and transcriptomic investigations** aims to contribute to the scientific basis for future crop improvement. O_3 effects on plant's metabolism are primarily induced by an increased production of reactive oxygen species (ROS), both outside and inside the plant cell, which is a common feature of biotic (pathogens, insects) and edaphic stresses (drought, high light, UV, cold…) in plants. This "oxidative burst" activates signal transduction pathways that influence plant defence responses and programmed cell death (PCD). Moreover, several stimuli that generate oxidative stress induce the expression of the same sub-set of defence genes and activate a PCD pathway that was also shown to be induced by superoxide anion and hydrogen peroxide treatment alone. This phenomenon suggests that distinct stresses may activate the same, or at least overlapping, signal transduction pathways involving salicylic and jasmonic acid and ethylene. As such, O_3 has been recognized as a good tool to study signaling cascades that involve apoplastic ROS formation in the regulation of gene expression and can be used to increase our understanding of the complex network of interacting signalling pathways involved in plant defence mechanisms (Rao *et al*, 2000).

In conclusion, this study aims to quantify the impact of predicted increases in tropospheric O_3 on yield and quality of *Brassica napus* and *Brassica oleracea* cv Italic to provide reliable O_3 dose response relationships for an integrated O_3 risk assessment with regard to food and feed security and safety. Changes in metabolism of antioxidants and GSLs and gene expression will contribute to the understanding of plant defence responses and signalling pathways in general. Increasing knowledge of the plant-environment and plant-pathogen interactions will surely provide novel strategies to stabilize agricultural yield and quality in a fluctuating environment (EPSO, 2005). It is also imperative to be able to understand and quantify the full impact of our changing environment, in order to identify the risks and justify the appropriate actions.

2 GENERAL METHODOLOGY

2.1 Controlled ozone exposure of Brassica species

2.1.1 Plant material: oilseed rape and broccoli

The choice for the *Brassica* species oilseed rape (*Brassica napus* L.) and broccoli (*Brassica oleracea* L. cv. *Italica*) was based on their importance for:

- Economy: production of rapeseed was 24.389 Mt, cabbage was 110.000 Mt in Belgium in 2005 (FAOSTAT data, 2006). Rapeseed is the third most important world source of vegetable oil (Lühs & Friedt, 1994). After removal of the oil, the residual rapeseed meal contains proteins (35-40%) with similar feed value of soya and is used as a feed supplement.
- Health: *Brassica* vegetables contain vitamins and GSLs that have potential value as cancer chemoproventive agents, but have also proven to cause toxic effects when fed to animals. Rapeseed oil has the lowest amount of saturated fatty acids of the vegetable oils (Orthoefer, 1996), these lipid profiles may have significant effects on obesity. Broccoli is one of the vegetables that are currently under investigation for its cancer-preventive properties.
- The environment: rapeseed oil can serve as a biofuel or as renewable resource for industrial applications. Moreover, *Brassicaceae* plants have tremendous potential to be used in crop rotations as a natural pesticide source, thereby decreasing our dependence on synthetic chemicals.

2.1.2 Preliminary testing in environmentally controlled chambers

Initially, in 2007, two short term, acute O_3 fumigations have been performed in 4 environmentally controlled chambers (1.5 x 2.2 x 2.4 m) located at VAR (Tervuren) (Figure 1).

Figure 1: Environmentally controlled chambers for O_3 exposure

These experiments aimed to determine the relative sensitivity of the species to be used in the main OTC experiment. At the same time the sampling protocol for the biochemical and molecular analyses and

measuring techniques for photosynthesis and fluorescence assessments were tested. The physiological measurements (photosynthesis, chlorophyll a fluorescence, chlorophyll content) were destined to identify differences in O_3 sensitivity between the cultivars. Each chamber contained four commercial cultivars of broccoli and oilseed rape, cultivated in pots, three plants per cultivar. The selection of these cultivars was mainly based on their relative importance for the Belgian market. All oilseed cultivars were double low varieties with low eruric acid and GSL content, suitable for human and animal consumption. For oilseed rape two spring cultivars (Ability & Simon) and two winter cultivars (Grizzly & Hydromel) were selected; for broccoli the cultivars Monaco, Montop, Fiesta & Lord were compared.
The short term fumigation experiment was repeated in 2008 at a more elevated O_3 exposure level and with only one cultivar per species (Ability and Monaco) to obtain more significant data on their physiological, biochemical & molecular responses to an acute O_3 exposure (Table I). Plants were approximately six weeks old at the time of fumigation.
In the chambers light was provided by Na-lamps (Son-T Agro 400W, Philips) in combination with Hg-lamps (HPI-T 400W, Philips) that can supply up to 300 µmol PAR (Photosynthetic Active Radiation) m^{-2} s^{-1} at plant level. Day/night regime (16/8 h), temperature (23/18°C) and air humidity (60/70 %) were controlled automatically. Two control chambers were ventilated with charcoal-purafil filtered air, so that the SO_2, NO, NO_2 and O_3 concentrations were reduced to < 15 ppb. In two other chambers, O_3 was added at the desired concentrations during 5h/day by means of mass flow controllers. O_3 was generated by electrical discharge from pure oxygen with an ozone generator (CMG 3-3, Innovatec, The Netherlands). O_3 concentrations were continuously monitored by UV absorption (41M, Environnement S.A, France); NO_x concentrations were measured by chemiluminescence (model 200A, API, USA). For more details on measurements and analysis: see 'Activity report for Intermediary Evaluation – Doc G', 'Annual Scientific report 2007' and chapter 2.2 and 2.4 of this document.

Table I: Overview of the fumigation experiments in closed chambers at VAR

Experiment	Data		O_3 conc		cv/species/	plants/cv/
	start	end	5 hr avg	max	chamber	chamber
Acute O_3 exp 1	19/03/07	23/03/07	96 ppb	139 ppb	4	3
Acute O_3 exp 2	02/04/07	06/04/07	110 ppb	184 ppb	4	3
Acute O_3 exp 3	23/04/08	27/04/08	145 ppb	197 ppb	1	18

2.1.3 Long term ozone exposure in Open-Top Chambers (OTCs)

In 2007, 2008 and 2009 oilseed rape and broccoli crops were exposed to different levels of O_3 for 8 hrs/day during their entire growth, from sowing or planting until harvest. The exposure was performed in 15 open-top chambers distributed over an area of approximately 30 ares (Figure 2).

Figure 2: Open-Top Chambers for long term ozone exposure of oilseed rape and broccoli

The objective of an open-top chamber design is to create an environment where air quality can be controlled, but where conditions are closer to those in the field than can be achieved in closed, environmentally controlled systems. It must however be recognized that it is impossible to replicate field conditions perfectly (Unsworth, 1986). Therefore six unchambered field plots (OPs) were included in the experimental set-up to compare the environment and plant growth between chambers and ambient air (chamber effect).

The OTCs have a decagonal ground plan (3 m diameter); the soil surface covers 7.3 m² and the chamber volume is 19.6 m³. The top of the chambers is not covered to prevent a greenhouse effect and to allow an unhindered flow-through of air. Unfiltered air is blown into the chambers by means of a ventilation unit and a perforated air duct suspended along the chamber wall. Per minute at least four air changes are accomplished at a flow rate of 1.36 $m^3\ s^{-1}$, allowing a homogenous air distribution. To prevent uncontrolled influx of ambient air, a frustum is placed on top of the chamber at a 40° downward angle.

Brassica napus cv Ability was sown in nine OTCs and three OPs (three blocks each containing an unfiltered air treatment (NF), two O_3 treatments NF+20 ppb O_3 (NF^+) & NF+40 ppb O_3 (NF^{++}) and one OP. The level of the O_3 additions was chosen in accordance with the IPCC scenarios on future increases in tropospheric O_3 levels. *Brassica oleracea* L. cv Monaco was sown and precultivated in the greenhouse. After five to six weeks the plants were transferred to six OTCs and three OPs (three blocks each containing an NF & NF^{++} treatment & one OP) at 50 cm spacing within and between the rows. To minimise individual chamber effects, the O_3 treatments were distributed differently over the individual OTCs during the three growing seasons (as far as this was technically feasible). O_3 was always added during eight hours per day, from from 11 am until 7 pm (local time) because ambient O_3 concentrations show strong diurnal cycles with daytime maxima (Figure 3). Peak concentrations occur mainly during the afternoon, when photochemical O_3 production is most active. Moreover, since O_3 uptake is dependent on stomatal opening this is also the time when the highest plant absorbance occurs.

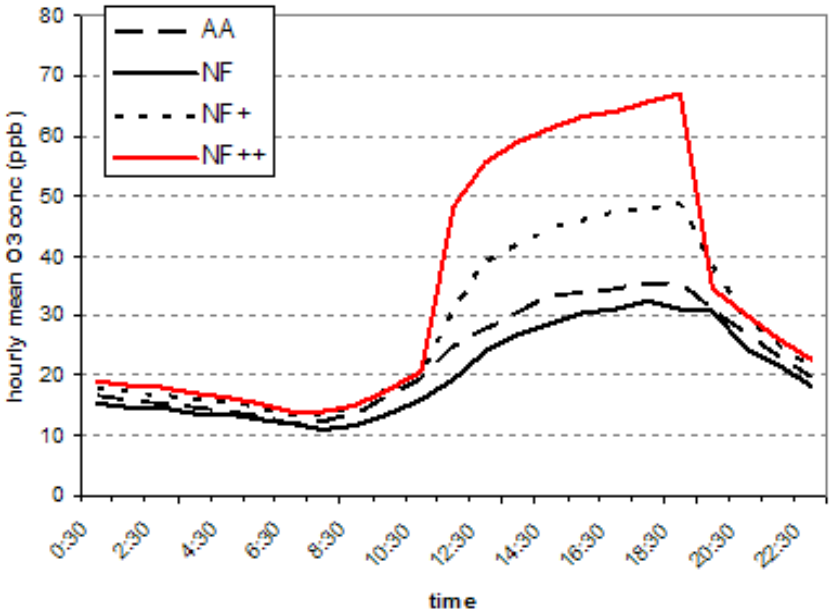


Figure 3: Diurnal profile of O_3 concentrations in ambient air (AA = measured on open-field plots OPs), non-filtered OTCs (NF) and OTCs with O_3 addition (NF+ & NF++)

O_3 was produced with an O_3 generator (CMG 3-3, Innovatec, The Netherlands) from pure oxygen by electric discharge. The supply of O_3 was adjusted with mass flow controllers (models 5850 TR, Brooks Instrument B.V., Fisher-Rosemount, The Netherlands) controlled by a microprocessor in reference to the actually measured O_3 concentrations. Climatic parameters (air and soil temperature, vapour pressure deficit (VPD), global radiation (GR), soil moisture potential and rainfall) were continuously monitored and stored as hourly averages (Table II).

Table II: Climatic variables in the open top chambers for the growing seasons of 2007, 2008 and 2009 for oilseed rape and broccoli. Mean values are given for air temperature, soil temperature, vapour pressure deficit and global radiation.

	Oilseed rape			Broccoli		
Variable	2007	2008	2009	2007	2008	2009
Air temperature (°C)	16.6	16.5	16.0	17.3	17.4	18.5
Soil temperature (°C)	16.3	15.6	15.1	16.6	16.4	17.4
VPD (kPa)	0.41	0.47	0.56	0.42	0.45	0.64
GR (MJ m^{-2} d^{-1})	11.8	14.3	14.4	12.8	14.1	15.6
8 hr avg [O_3] NF (ppb)	30	33	32	29	29	32
8 hr avg [O_3] NF+ (ppb)	46	53	48			
8 hr avg [O_3] NF++ (ppb)	63	73	63	58	63	66

NF: Non Filtered air: NF+, NF++: Non Filtered air with additional O_3; VPD: Vapour pressure deficit; GR: Global radiation; avg: average

Table III: Overview of crop cultivation, treatments and yield quantity + quality data in 2007, 2008 and 2009

	Brassica napus cv Ability	*Brassica oleracea* cv Monaco
Sowing date	sown in full soil 18/04/2007 10-11/04/2008 01/04/2009	sown in climate chamber 02/05/2007 07/05/2008 05/05/2009
50 % emergence /planting date	09/05/2007 21/04/2008 11/04/2009	13/06/2007 04/06/2008 08-09/06/2009
Plant density	120 pl/m²	4 pl/m²
Harvest	6-9 subplots (1m) per chamber 21/08/2007 08/08/2008 04/08/2009	12 plants/chamber 07/08/2007 5-6/08/2008 17-18/08/2009
Yield quantity	Seed yield, 1000 seed weight, seed volume/weight	FW + DW of leaves, stems & inflorescence
Yield quality	Seeds : Fatty acids GSLs Proteins Vitamins	Florets GSLs Proteins vitamins
O_3 fumigation	2007* : 25/05- 10/08 2008 : 07/05- 08/08 2009 : 15/04- 30/07	13/06/2007 - 07/08/2007 09/06/2008 – 06/08/2008 12/06 – 18/08/2009
Treatments	3 NF = unfiltered air 3 NF^+ = NF + 20 ppb O_3 3 NF^{++} = NF + 40 ppb O_3 3 OP = open field plots	3 NF = unfiltered air No NF^+ 3 NF^{++} = NF + 40 ppb O_3 3 OP = open field plots

* start of fumigation was delayed due to technical problems

Before sowing or planting the plots were fertilized according to the recommendations of the "Bodemkundige Dienst van België" based on previous soil analysis. Appropriate fungicides, insecticides or molluscicides were applied to protect the crop against diseases and insect or snail pests. When necessary, belowground irrigation was provided. At harvest 6 to 9 subplots of oilseed rape of 1 m row length were harvested per chamber. The seeds were threshed at the APPO (Association pour la Promotion des Protéagineux et des Oléagineux) in Gembloux. Seed samples were purified and stored for further quality analysis at VAR. For broccoli 12 plants per chamber were harvested. Fresh marketable yield, i.e. the flower bud with the upper part of the stem, was weighed immediately after harvest. Fresh samples for quality analysis were immediately frosen in liquid nitrogen and stored at -80°C for further biochemical and molecular analyses. Total above-ground biomass was determined after drying at 75°C, until constant weight was achieved. Details on crop cultivation and treatments are given in Table III.

2.2 Assessment of physiological plant performance and crop growth

At the end of the closed chamber experiment and throughout crop growth in the OTC experiments, physiological assessments of plant performance were carried out. The main objective of these measurements was to identify the extent to which O_3 was causing a physiological stress response and to relate these events to quantitative and qualitative yield effects and biochemical changes at the leaf level.

Figure 4: Photosynthetic measurements on broccoli in Open-Top Chambers

Following measurements have been performed:

- Light saturated photosynthesis (A_{sat}), stomatal conductance (g_s) and the actual quantum yield of photochemistry ($\Phi_{PSII} = F'_v/F'_m$) were measured with a portable gas exchange system (Li-6400, Li-cor Inc., USA) equipped with a fluorescence chamber (Li-6400-40 leaf chamber fluorometer, Li-cor inc., USA) (Figure 4). These data were recorded after allowing the leaves to reach steady state conditions (between 10 and 15 minutes) at saturating photosynthetically active radiation (PAR = 1600 µmol m^{-2} s^{-1}) and at a CO_2 concentration of 380 ppm and ambient temperature and humidity.
- The maximum quantum yield of Photosystem II (PSII) (F_v/F_m) was measured on five oilseed rape plants and three broccoli plants per chamber by means of the non-modulated Plant Efficiency Analyser (Handy PEA, Hansatech Ltd., UK) after dark acclimation for 15 minutes. The Handy-PEA software allows extracting the fluorescence value at five defined time points: T1= 50 µsec, T2= 100 µsec, T3= (K step) 300 µsec, T4 = (J step) 2 msec and T5 = (I step) 30 msec. These fluorescence values are used to derive the Performance Index (P.I.) which gives a relative estimate of the overall energy flow in PSII (Strasser *et al*, 1998).

- Chlorophyll concentration with SPAD-502 (Minolta, Japan). These SPAD values are recalculated to total chlorophyll content using a calibration curve determined by spectrophotometric analysis of leaf extracts at 664 nm and 647 nm. Therefore leaf fragments from a range of leaf ages were incubated with dimethylformamide (24 h, 4°C). The chlorophyll concentration was calculated according to Porra (2002). The calibration curve used for oilseed rape leaves was [chlorophyll (µg/g fresh weight (FW))] = 0.497 $SPAD^2$ + 1.180 SPAD + 156.110 (R^2 = 0.88) and the calibration curve used for broccoli leaves was [chlorophyll (µg/gFW)] = 0.290 $SPAD^2$ + 7.698 SPAD + 30.957 (R^2 = 0.70) (see also 3.1.3 - Figure 9).
- Leaf area index (LAI) with the LAI-2000 Plant Canopy Analyser (Li-Cor, USA), averaged over three measurements in each OTC. Maximum leaf area (MLA) was determined when the LAI reached its highest level.

All measurements were made on the youngest fully developed leaf, for certain assessments also a labelled lower canopy leaf was monitored during its further development.

2.3 Ozone flux modelling

Historically, critical levels of O_3 for vegetation were based on the concentration of O_3 in the atmosphere, but it has long been recognised that plant responses to O_3 are more closely related to the internal O_3 dose in the leaf, or the instantaneous flux of O_3 through the stomata, than the ambient O_3 exposure (e.g. Lefohn and Runeckles, 1987; Fuhrer, 2000). This approach requires mathematical modelling of the pathway of O_3 into the leaf including atmospheric, boundary layer and stomatal resistances (Vandermeiren *et al*, 2009).

The key process in relating O_3 exposure to biochemical, physiological and final yield responses is the ease with which O_3 gains access into the stomata. The actual diffusion of gasses through the stomata, expressed by the stomatal conductance (g_s), is proportional to the atmospheric concentration of O_3, but is strongly controlled by the stomatal aperture. Unlike O_3 concentrations, stomatal O_3 uptake is hard to measure in the field. Because stomatal O_3 fluxes are partially determined by stomatal aperture, the calculation of stomatal O_3 fluxes requires models that accurately predict stomatal conductance (Op de Beeck *et al*, 2010). The influence of plant phenology, irradiance, temperature, air humidity and soil moisture on stomatal O_3 uptake is incorporated in the models through their interference with g_s (Jarvis, 1976).

The gas exchange measurements were used for parameterization and validation of two distinct O_3 flux algorithms. These algorithms simulate oilseed rape and broccoli stomatal O_3 fluxes for all fumigation regimes, as stomatal flux is the physiologically relevant O_3 exposure metric to which measured biochemical and ecophysiological plant ozone responses may be linked. Both algorithms calculate stomatal fluxes from measured O_3 concentrations and simulated stomatal conductance to O_3.

2.4 Leaf analysis: biochemical composition and gene expression

As explained before, O_3 exposure can induce several antioxidative defence pathways which may influence secondary metabolite concentrations in the plants. Biochemical changes at the leaf level may provide a first indication (biomarker) of oxidative stress. We concentrated our efforts mainly on those compounds that also represent an important quality parameter in the consumable end products namely GSLs and the antioxidants ASC (vit C) and α- TOC (vit E). GSH is another important antioxidant interacting with the ASC pathway and is easily followed in the same HPLC assay. As also other antioxidants possibly change under enhanced O_3 levels (D'Haese *et al*, 2005) and therefore the total

antioxidative capacity of the samples was assessed. We also includes antioxidative enzyme activity in out evaluation of the plant's defence mechanism. Some samples were used for RNA extraction, to examine expression levels for O_3 affected pathways. In the preliminary tests in the closed chambers leaf samples were taken at the end of the experiment when the plants were about six weeks old. In the OTCs leaf sampling took place before (both species) (S1) and shortly after flowering (oilseed rape) or near harvest (broccoli) (S2).

2.4.1 GSL analysis

Since not only the total GSL content is of importance, also the individual components have been determined (e.g. progoitrin, glucoraphine, gluconapin, glucobrassicin etc.). Fresh samples (at least 10 g/sample) were homogenised and immediately frozen in liquid nitrogen, stored at -80°C and the extraction was performed as soon as possible. Analysis was performed according to the (slightly modified) International Standard ISO 9167-1 (1992). For oilseed samples, 200 mg of air dried material was used; for green plant material 1 g was used. The samples were ground in liquid nitrogen, extracted with methanol at 70-75°C and an internal standard (2 µmol sinigrine or glucotropaeoline) was added. After centrifugation the supernatant was dried in a rotavapor, resuspended with distilled water and filtered. The extract was transferred to an ion-exchange column of DEAE Sephadex A25, activated with imidazol formate and pH was adjusted with sodium acetate buffer (pH 4). The extract was desulphated overnight with a sulphatase solution of *Helix Pomata* and subsequently the desulfoGSLs were eluated with water. The GSLs were separated by means of HPLC (Alliance 2695, Waters, USA) over a C18 column (AtlantisTM dC18, 3 µm, Waters, USA) at 30°C and wavelength of 229 nm. The mobile phase is a gradient from 0 to 40% acetonitrile in 30 min at a flow rate of 0.5 ml/min. Identification of the chromatographic peaks is based on the retention time and photodiode array profile (Waters 2996, USA) in comparison to a library obtained from analysis of commercially available pure standards. Quantification is made in reference to the internal standard and response factors found in literature (Wathelet *et al*, 2004) and according to ISO 9167-1 (1992). Efficiency of the extraction and analysis is checked in reference to certified reference material ERM® - BC 190 and ERM® - BC 366 (IRMM, Belgium). Results are expressed as µmol/g air dried material for oilseeds (<10% moisture) and oven dried weight for all other plant tissues.

2.4.2 Ascorbate (ASC) , glutathione (GSH) and α-tocopherol (TOC) analysis

For all biochemical analyses described below, fresh plant material (+/-100 mg) was used. For determination of ASC and GSH concentrations, samples of all three growing seasons were harvested, snap frozen in liquid nitrogen and stored at -80°C. Frozen samples were ground using glass beads and a Magnalyzer (Roche Applied Science). ASC and GSH were extracted in an acidic environment by addition of ice-cold metaphosphoric acid (6%, 0.5 ml). The samples were clarified by centrifugation at 16 000g at 4°C for 20 minutes. Antioxidants were separated on a 100 mm x 4.6 mm Polaris C18-A reversed phase HPLC column (3 µm particle size; 30°C; Varian, CA USA) with an isocratic flow of 1 ml/min of the elution buffer (25 mM KPO_4-buffer, pH 3.00). The components were quantified using a homemade electrochemical detector with glassy carbon electrode and a Schott pt 62 reference electrode (Mainz, Germany). The purity and identification of the peaks was confirmed with a diode array detector (SPD-M10AVP, Shimadzu, Hertogenbosch, The Netherlands), placed online with the electrochemical detector. As only the reduced ASC (ASC) and GSH (GSH) was measured, the amount of total ASC (tASC) and GSH (tGSH), both the reduced and oxidized form, was measured in a DTT

reduced fraction. Reduction of the sample was obtained by incubation of an aliquot of the extract in 400 mM Tris and 200 mM DTT for 15 min in the dark.
For α-TOC determination an organic extraction is performed. Samples were again ground using glass beads and a Magnalyzer (Roche). To the powdered extracts γ-TOC was added as internal standard (100 µl, 20 µg/l) together with 1 ml of hexane and samples were shaken using the Magnalyzer device. After brief centrifugation the eluens was collected, subsequently the samples were re-extracted in 1 ml of hexane, in total 4 ml of hexane was used to extract all α-TOC. The combined fraction was subsequently filtered through a PVDF filter (0.45 µm), concentrated under vacuum, and separated on a 250 mm x 4.6 mm Partical PAC 5u HPLC column (5 µm particle size; 40°C; Alltech, Il USA) with an isocratic flow of 1.5 ml/min of the elution buffer (hexane, 8% Tetrahydrofuran). The concentration of α-TOC was determined with the fluorescence detector (ex 290 nm, em 330 nm, RF10, Shimadzu, Hertogenbosch, Netherlands) after comparison with the internal standard. The purity and identity of the peaks was confirmed with a diode array detector (SPD-M10AVP, Shimadzu, Hertogenbosch, The Netherlands) online with the fluorescence detector.

2.4.3 Total (water soluble) antioxidative capacity (TAC)

Samples were ground with the Magnalyzer (Roche) as described for ASC en GSH. They were kept frozen until addition of the acidic extraction medium (0.01 N HCl) and afterwards treated below 4°C. Samples were clarified by centrifugation at 16000 g for 20 minutes. TAC was measured as the capacity of andioxidants to reduce the complex Fe^{3+}-TBTZ to Fe^{2+}-TBTZ. The latter being a blue coloured product that can be measured at 600 nm after 10 min. A standard curve was made using TROLOX, a vitamin E analogue, in a concentration range between 250 and 20 µM and therefore TAC was expressed as µmol TROLOX equivalents/g FW.

2.4.4 Enzymatic activity of dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), ascorbate peroxidase (APX), superoxide dismutase (SOD) and unspecific peroxidase (POX)

These analyses were only performed on leaf samples taken in 2008. Samples of 200 mg FW were extracted in 1 mL of a MES/KOH buffer (pH 6.0; 40 mM KCl; 2mM $CaCl_2$; 1mM ASC). All enzyme activities were determined by kinetic reactions at 25 °C in 200 µL, using a micro-plate reader. APX, DHAR, MDHAR and GR activities were measured according to Murshed *et al* (2008). APX activity was measured in a reaction mixture of 50 mM potassium phosphate (pH 7.0), 0.25 mM ASC, 10 µL extract and 5 mM H_2O_2. Activity was determined by measuring the decrease in absorbance at 290 nm and recalculated with an extinction coefficient of 2.8 mM^{-1} cm^{-1}. DHAR activity was assayed in a reaction mixture of 50 mM HEPES buffer (pH 7.0), 0.1 mM EDTA, 2.5 mM GSH, 0.2 mM DHA and 10 µL extract. Activity was determined by measuring the increase in absorbance of ASC at 265 nm with an extinction coefficient of 14.0 Mm^{-1} cm^{-1}. MDHAR activity was determined in a reaction mixture of 100 mM HEPES buffer (pH 7.6), 2.5 mM ASC, 0.25 mM nicotinamide adenine dinucleotide (NADH), 10 µL extract, and 0.4 unit ASC oxidase. Activity was determined by measuring the decrease in absorbance at 340 nm of NADH and recalculated with a 6.22 Mm^{-1} cm^{-1}extinction coefficient. GR activity was determined in a reaction mixture with 100 mM HEPES buffer (pH 8), 0.5 mM EDTA, 0.25 mM NADPH, 10 µL extract, and 20mM GSSG. Activity was determined by measuring the decrease in absorbance at 340 nm of NADH with a 6.22 mM^{-1} cm^{-1} extinction coefficient. SOD activity (Beauchamp and Fridovich, 1971) was determined according to Dhindsa *et al* (1981). SOD activity was assayed in a reaction mixture containing 50 mM potassium phosphate (pH 7.8) buffer, 13 mM methionine, 75 mM nitro blue

tetrazolium (NBT), 0.1 mM EDTA, 10 µL of extract and 2 mM riboflavin. The reaction was carried out at 25 °C under high light for 5 min. The reaction was stopped by switching off the light and covering the tubes. A blank measurement was performed with samples that did not receive any light. The absorbance of NBT was measured at 560 nm using spectrophotometer. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate NBT chloride photoreduction. POX activity was assayed according to the method of Kumar and Khan (1982). The assay mixture of POX contained 0.05M potassium phosphate (pH 6.8), 0.01 M pyrogallol, 10 µl of 20 µL of enzyme extract and 0.01 M H_2O_2. Activity was determined by measuring the decrease in absorbance of pyrogallol at 430 nm with an extinction coefficient of 2.47 mM^{-1} cm^{-1}.

2.4.5 Transcriptomic analysis

RNA was extracted with the Concert Plant RNA reagent (Invitrogen, USA) protocol on leaves sampled near the end of the vegetative stage (S1) and during the generative phase (S2) shortly after flowering (oilseed rape) or at harvest (broccoli). The manufacturer's protocol was slightly adjusted with an additional CTAB-cleaning step. After chloroform clarification, 0.325 ml of CTAB (2%) was added. After an incubation period of 10 minutes at 60°C, isopropylalcohol was added and the protocol was continued as described. The quantity of RNA was determined spectrophotometrically (Nanodrop, Nanodrop technologies) and the quality was controlled using a gel electrophoresis separation method (Qiaxcel, Qiagen). Total RNA was pooled and reversed transcribed to first strand cDNA using Superscript III Reverse transcriptase kit (Roche). Expression levels of specific interesting genes were additionally determined using quantification with the real-time PCR (RT-PCR) technique. The actual RT-PCR reactions were performed on several genes of interest using the LightCycler® FastStart DNA Master PLUS SYBR Green I kit (Roche) according to the manufacturer's description. SYBR Green is the fluorochrome that will incorporate into new DNA strains generated during the PCR reaction and that enables quantification. Afterwards calculation of the expression levels compared to house-keeping genes (UBQ10) was performed as described earlier (Pfaffl, 2001).

2.5 Quality analysis of marketable end products

Besides the carbohydrate and protein, also the oil content and fatty acid composition of seeds are an important quality aspect particularly for oil producing crops such as soybean (*Glycine max*), oilseed rape (*Brassica napus*), peanut (*Arachis hypogaea*), mustard (*Brassica campestris*) e.a.. In general, there is an inverse relationship between seed oil and protein content but environmental factors may alter the seed oil:protein ratio. Oilseed rape is the third most important world source of vegetable oil (Lühls & Friedt, 1994) whilst the protein content of the residual seed meal is similar to soya and used as feed supplement. Moreover, the seeds are rich in linoleic and linolenic acids, essential fatty acids and precursors of the Omega 6 and Omega 3 fatty acid families. An important health-related quality parameter for most *Brassicaceae* is their GSL content. These compounds possess a wide range of antifungal, antibacterial and antimicrobial activities and have been attributed anti-carcinogenic properties (Talalay and Fahey, 2001). In animal feed however, they decrease digestibility and may cause goitre and haemolytic anaemia if supplemented at excessive rates (Stoewsand, 1995).
On the seeds of oilseed rape following quality analysis were performed:

- GSL content: method see 2.4.1
- Protein concentration: determined by near infra-red analysis at the Centre Wallon de Recherches Agronomiques (Gembloux, Belgium).

- Fatty acid content: performed at the Canola Analytical Laboratory of Bayer CropScience (Saskatoon, Canada). The types and amounts of fatty acids are determined by extracting the oil from crushed seed, converting the oil to fatty acid methyl ester (FAME) derivatives using sodium methoxide dissolved in methanol, and analyzing the FAME's by capillary gas-liquid chromatography using a flame ionization detector. Each fatty acid is reported as a weight percentage based on the total amount of fatty acids present. The method is suitable for the analysis of FAME's having 8 to 24 carbon atoms at levels ranging from about 0.05% to 100% on a relative weight basis.
- TOC content (vit E): α, β, γ, δ – Tocopherol concentrations were determined at TNO (Zeist, The Netherlands). The crushed seeds were saponified in duplicate under reflux conditions for 30 min in 1.5 mol/l ethanolic potassium hydroxide, to which sulphide and sodium ascorbate were added. After cooling at room temperature, the samples were extracted with diisopropyl ether. Afterwards the extract was washed three times with an aqueous solution and subsequently, this purified extract was analyzed for α-, β-, γ-and δ-TOC with straight phase HPLC and fluorescence detection. Total vitamin E content of the seeds was calculated as the sum of α-, γ- and δ-TOC since β-TOC remained below the detection limit of 0.3 mg/100 g. Data are expressed in mg /100 g air dried seeds.

Quality analyses of broccoli vegetables included:

- GSL content: method see 2.4.1
- Protein concentration: the crude protein content of broccoli vegetables and all other plant material was calculated by multiplying the nitrogen content by 6.25 (EuropaBio, 2001). Total nitrogen was determined by the Kjeldahl method after digestion with suphuric acid and addition of Se reagent mixture.
- Antioxidants GSH, vitamin C (ASC) and vitamin E (α-TOC) content: method see 2.4.2

3 RESULTS

3.1 Results of the short acute O_3 exposure in environmentally controlled chambers

The aim of the short term exposure of oilseed rape and broccoli seedlings to an acute O_3 concentration fumigation experiments in the climatically controlled chambers was mainly twofold:

- To determine differences in O_3 sensitivity between broccoli and oilseed rape cultivars based on their physiological responses (photosynthesis, chlorophyll fluorescence, chlorophyll concentration)
- To test and establish protocols for biochemical and RNA sampling and analyses of leaves of oilseed rape and broccoli

For methodology: see 2.1.2

3.1.1 Differences in cultivar sensitivity to O_3

Due to space limitations, we were only able to screen four cultivars per species. The acute O_3 exposure did induce changes in photosynthetic performance but as ANOVA analysis did not indicate significant cultivar x O_3 interactions we could not identify any highly sensitive or tolerant cultivars (Figure 5). O_3 did seem to have a more detrimental impact on the photosynthetic performance of spring cultivars Ability and Simon as compared to the winter varieties Grizzly and Hydromel. Ability was the only oilseed rape cultivar that showed a significant decrease of F'_v/F'_m and SPAD in response to the O_3 fumigation. There were neither any significant cultivar x O_3 interactions with regard to the antioxidant and GSL response. Leaves of winter oilseed rape cultivars did have a higher GSL concentration in comparison to the spring cultivars. The youngest, fully developed leaves of oilseed rape mainly contained progoitrin, gluconapin and glucobrassicanapin. The major GSL in broccoli leaves was glucobrassicin (Vandermeiren *et al*, 2009).

Brassica napus L.

Brassica oleracea L. cv Italica

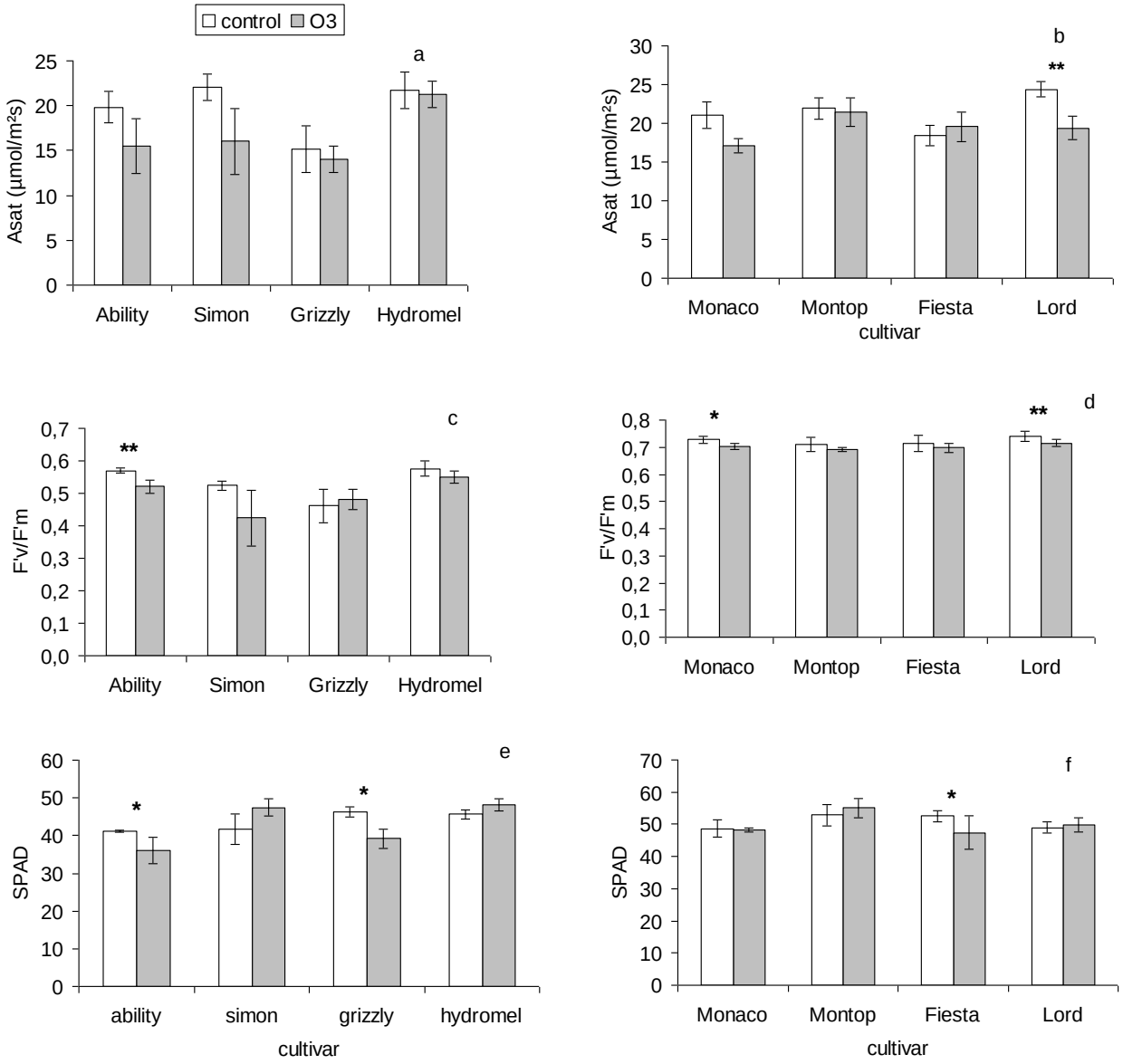


Figure 5: O_3 effect on light saturated CO_2 assimilation (A_{sat}) (a,b), actual photochemical efficiency (F'v/F'm) (c,d) and chlorophyll content (SPAD) (e,f) of oilseed rape (a,c,e) and broccoli cultivars (b,d,f). Significance of the difference with NF has been determined by ANOVA (*p < 0.10; ** p < 0.05)

3.1.2 Responses of spring oilseed rape (cv Ability) and broccoli (cv Monaco) to a short acute O_3 episode

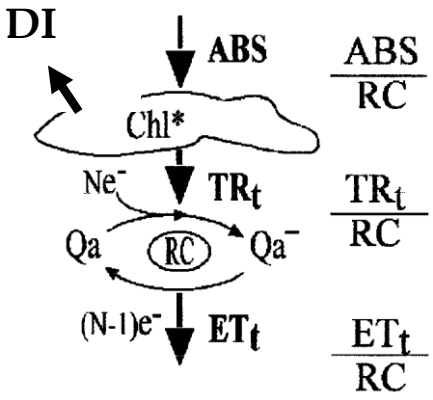
The closed chamber fumigation experiment was repeated in April 2008 with only one cultivar of each species and at a slightly higher O_3 level (150 instead of 100 ppb) to increase the statistical significance of the physiological assessments and of the biochemical and molecular analyses.

Figure 6: Leaf necrosis and chlorosis on *Brassica napus*, induced by acute O_3 exposure

Contrary to the previous experiment, visible injury symptoms were observed on O_3 treated *Brassica napus* (Figure 6) and the percentage of flowering plants was increased.

The efficiency of photosystem II was assessed by chlorophyll fluorescence measurements after dark adaptation. The potential photochemical efficiency is determined as F_v/F_m. To quantify the energy fluxes in the photosynthetic apparatus the PEA data were further analysed according to the theory of Strasser:

ABS = flux of photons absorbed by the antenna pigments Chl*
DI = dissipation energy flux at the level of the antenna chlorophylls
TR = energy trapped by the reaction centre and converted into redox energy by reducing QA
ET = electron flux further downstream leading to CO_2 fixation
RC = reaction centre
CS = cross section
RC/CS = density of the reaction centers
PI = vitality performance index = performance of the overall energy flow (ABS/CS*TR/CS*ET/CS)



Both plant species showed the same relative O_3 response with regard to these parameters: the potential photochemical efficiency (F_v/F_m) was slightly decreased, but the performance index (PI) and number of active photochemical reaction centres per leaf cross section showed an even more pronounced decrease (Figure 7). There was also a tendency for an increase in energy dissipation meaning that a

larger proportion of the light energy was re-emitted as heat or fluorescence radiation instead of being efficiently used for photosynthesis.

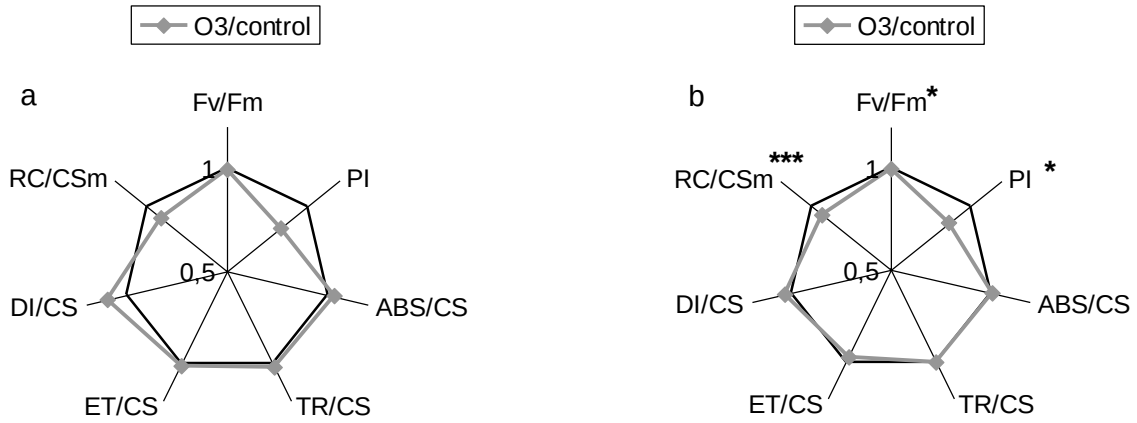


Figure 7 : Ratio of fluorescence parameter value of the O_3 treated versus control plants of oilseed rape (a) and broccoli (b). * and *** indicate a 90 and 99% sign O_3 effect (GLM)

There was a tendency for TAC to increase in broccoli leaves as a consequence of the acute O_3 treatment ($p = 0.09$). The contribution of TOC to TAC was determined to be 2,2% for *Brassica oleracea* and 5,6% for *Brassica napus*, no significant differences were observed here.

The short acute O_3 exposure caused a significant decrease of the total GSL content in broccoli leaves of cv Monaco, mainly due to the reduction of the indol GSLs glucobrassicin and neo-glucobrassicin (Figure 8).

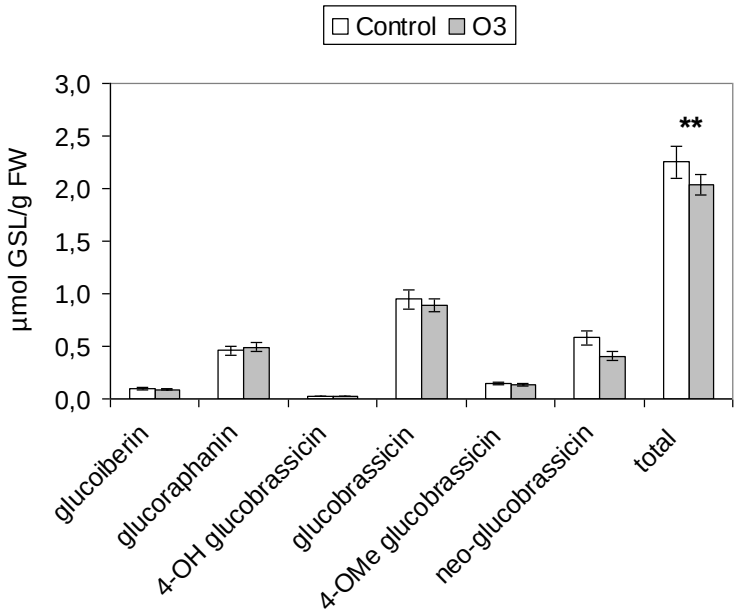


Figure 8: Effect of a short acute O_3 exposure GSL concentration in leaves of *Brassica oleracea* cv Monaco (** $p < 0.05$).

3.1.3 Testing of sampling and analytical procedures

- Both for the physiological and biochemical assessments we observed a large variation within each population, despite the highly controlled climatic conditions under which the plants were grown. This was an indication that a high number of replicate measurements would be needed to obtain reliable results in the OTC experiments.
- Older leaves contained much lower quantities of antioxidants and GSLs so it was decided to restrict the analyses to mature, but non-senescent upper canopy leaves in the field trials
- The protocol for RNA extraction was optimized. The use of primers designed for RT-PCR experiments with *Arabidopsis*-plants was examined.
- The destructive determination of the leaf chlorophyll content yielded following calibrations curves that can be used to recalculate the SPAD values (Figure 9). It became obvious that for broccoli SPAD values above 55 could not be reliably correlated with the true chlorphyll content.

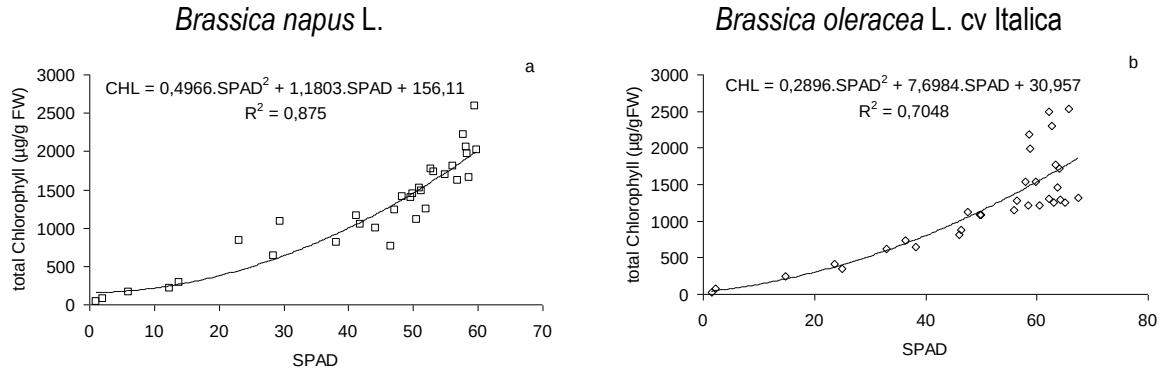


Figure 9: Relationship between the undestructive measurements of chlorophyll concentrations (a+b) of oilseed rape (a) and broccoli leaves (b) with the Minolta SPAD-502 meter and the spectrophotometric assay from extracts of leaf discs.

3.2 Comparison of two stomatal conductance models for ozone flux modeling using data from oilseed rape and broccoli (Op de Beeck *et al*, 2010).

There is general agreement within the expert community that the O_3 exposure metric to be used for the evaluation of plant response is preferably based on stomatal O_3 flux (Ashmore *et al*, 2004), as the sites of phytotoxic O_3 action are inside the plant and O_3 enters the plant through stomata (Pell *et al*, 1997). The calculation of stomatal O_3 fluxes on the regional scale requires stomatal models which are easily parameterized and accurately simulate stomatal conductance from a few driving environmental variables. Currently, a multiplicative Jarvis-type stomatal model (Jarvis, 1976; Mills, 2004) is applied in the ozone deposition algorithm (DO_3SE; Emberson *et al*, 2000) that makes part of the atmospheric chemical transport model used to predict the formation, transport and deposition of O_3 over Europe (EMEP; Tuovinen *et al*, 2004). For crops, the multiplicative stomatal model within DO_3SE has been parameterized for wheat and potato (Mills, 2004). There is a need, however, to extend this parameterization to other crops, like oilseed rape and broccoli. In addition, stomatal O_3 flux calculations for O_3 risk assessment are exclusively performed with the multiplicative stomatal model, although other suitable stomatal models are available. Of particular interest are so-called semi-empirical stomatal models, such as the Ball-Woodrow-Berry model (Ball *et al*, 1987) and the Ball-Berry-Leuning model (Leuning, 1995). Semi-empirical models are widely applied in plant growth and gas exchange modelling

(e.g., Pitman, 2003; Kramer *et al*, 2002). They simulate stomatal conductance directly from photosynthesis and can be fully environment-driven when coupled with a photosynthesis model. The aim of this section is to parameterize, validate and compare a multiplicative stomatal model and a semi-empirical coupled stomatal-photosynthesis model using data from the two *Brassica* species oilseed rape and broccoli. After a good parameterization of O_3 fluxes is established, these parameters can be used to effectively calculate POD_x values, which is the cumulative O_3 uptake, possibly with a threshold. Dose response relationships, both as a function of accumulated O_3 exposure and uptake, are described in 3.3.

3.2.1 Parameterization

One of the problems for a good parameterization was mainly due to the relatively fast light fluctuations that may occur in the field - particularly under partially cloudy skies. Because of the slow stomatal response to environmental changes, it is very unlikely that the point measurements of gas exchange were all made on leaves in full equilibrium with the level of photosynthetic radiation recorded at that particular moment. The two models we want to compare, however, simulate steady-state stomatal conductance. Large deviations from the steady state in the point measurements used for model parameterization and validation might therefore confound our results. To ensure that all data used for parameterization and validation at least approximate the steady state, we made a selection based on an empirical PAR criterion. Only those point measurements were selected for which instant measured PAR lay with the range of 20% each side of the hourly averaged measured PAR for the hourly period preceding the measurement. This tolerance range was arbitrarily chosen. After selection, 738 and 564 data points remained for oilseed rape and broccoli, respectively.

The multiplicative model was originally developed by Jarvis (1976) and later reformulated for the calculation of stomatal O_3 fluxes (Emberson *et al*, 2000; Mills, 2004). The model simulates stomatal conductance to O_3 (g_{st}) from a maximum value under optimal conditions (g_{stmax}), which is multiplied with dimensionless functions describing the relationship between g_{st} and individual driving variables:

$$g_{\mathrm{st}} = g_{\mathrm{stmax}} f_{\mathrm{phen}} \max\left\{ f_{\mathrm{min}}; f_{\mathrm{PAR}} f_{\mathrm{T}} f_{\mathrm{VPD}} \right\} . \qquad \text{Eq. 1}$$

Here f_{phen}, f_{PAR}, f_T, and f_{VPD} are functions accounting for the effect of phenology, PAR, T, and VPD, respectively. All functions range between 0 and 1. The parameter f_{min} represents night-time g_{st}, expressed as a fraction of g_{stmax}. A function for the effect of soil humidity was not included in the model, because plants were irrigated and did not suffer from soil water stress.

The coupled model combines a semi-empirical stomatal model with the biochemical photosynthesis model of Farquhar (Farquhar *et al*, 1980). In this study, we used a modified version of the Ball-Berry-Leuning stomatal model (Leuning, 1995), as applied in Yu *et al* (2001) and Uddling *et al* (2005):

$$g_{\mathrm{st}} = g_0 + a_1 0.96 \frac{A_{\mathrm{n}} + R_{\mathrm{d}}}{C_{\mathrm{s}} - \Gamma} f_{\mathrm{VPDc}} \qquad \text{Eq. 2}$$

Here g_0 is g_{st} in the dark, a_1 is an empirical scaling parameter, A_n is net photosynthesis, R_d is leaf respiration rate, C_s is the leaf surface CO_2 concentration, Γ is the CO_2 compensation point, and f_{VPDc} is a dimensionless VPD function ranging between 0 and 1. The factor 0.96 converts stomatal conductance for CO_2 to stomatal conductance to O_3.

The g_{st} values in function of the different environmental parameters are given in Figure 10. Also the fitted functions from the multiplicative model are shown. As noticed, these functions are not exactly on the boundery of the data points. This was necessary to obtain a good fit between measured and obtained g_{st}. It is explained by the fact that we had to account for measurements that were not taken under steady state conditions, also $g_{st\ max}$ was estimated lower at first, but adjusted to a value of 0.55 mol m^{-2} s^{-1}. Table IV shows the optimized and fixed values for both models. In the coupled model, the photosynthesis model used mainly parameters which were taken from the literature (values not shown): k_{RdVm25}, f, a_{abs}, O, R, K_{c25}, K_{o25}, Γ'_{25}, ΔH_{aKc}, ΔH_{aKo}, $\Delta H_{a\Gamma'25}$, ΔH_{aRd}, ΔH_{aVm}, ΔH_{aJm}, ΔH_{dVm}, ΔH_{dJm}, ΔS_{Vm}, ΔS_{Jm} (Jones, 1992; de Pury *et al*, 1997; Leuning, 2002; Müller and Diepenbrock, 2006)

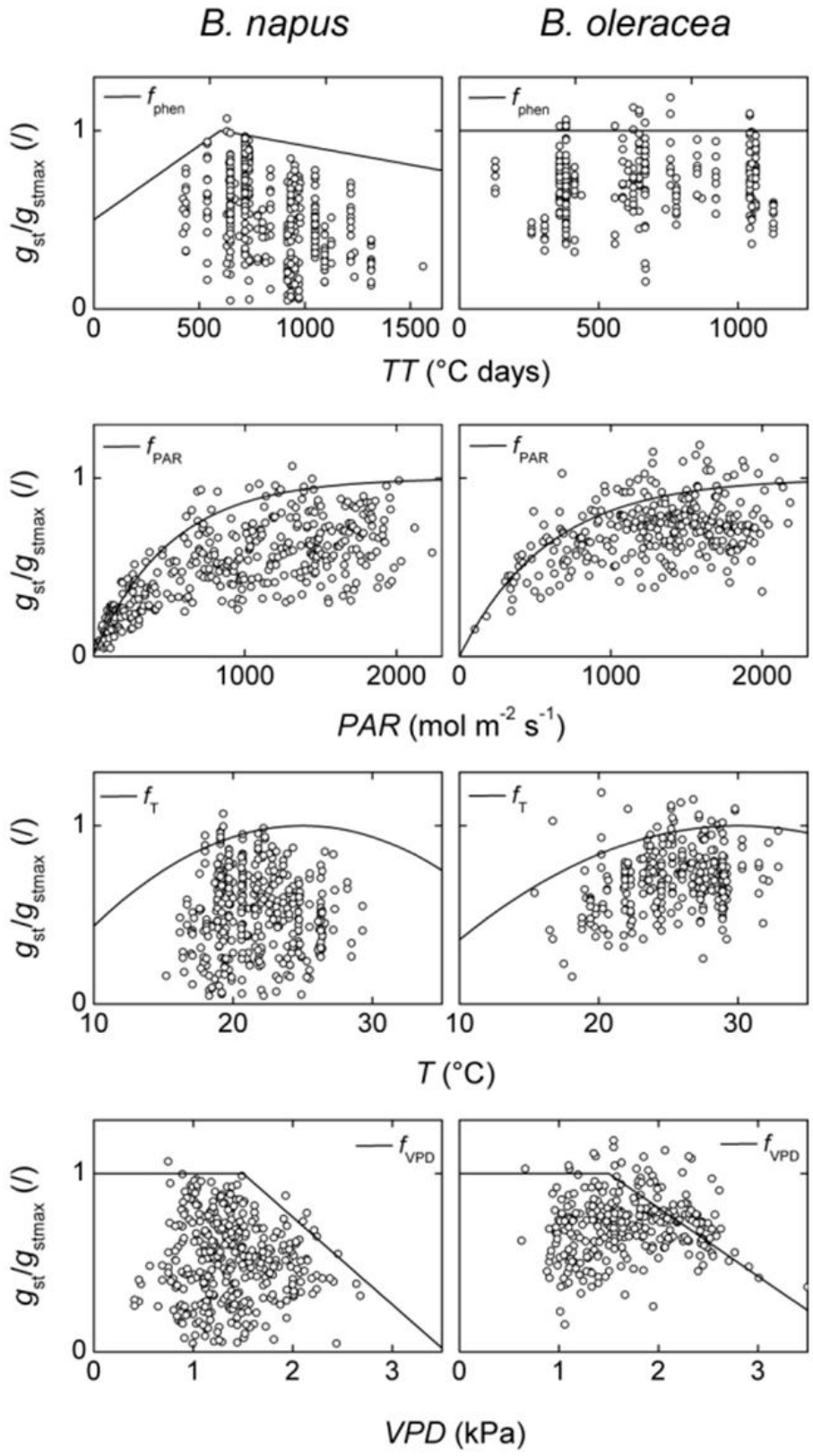


Figure 10: Parameterization of the multiplicative model for oilseed rape (*B. napus*) (left graphs, *n*=369) and broccoli (*B. oleracea*) (right graphs, *n*=290). Plots of relative stomatal conductance (g_{st}/g_{stmax}) versus thermal time (*TT*), photosynthetically active radiation (*PAR*), air temperature (*T*), and air vapour pressure deficit (*VPD*). Solid lines are the curves of the dimensionless functions fitted.

Table IV: List of input values of model parameters for oilseed rape (*B. napus*) and broccoli (*B. oleracea*), with their reference.

parameter	value		units	reference
	B. napus	*B. oleracea*		
multiplicative model				
g_{stmax}	0.55	0.7	mol m^{-2} s^{-1}	fixed (*B. napus*), optimized (*B. oleracea*)
f_{min}	0.02	0.04	dimensionless	optimized as g_0/g_{stmax}
α_{PAR}	0.0020	0.0017	dimensionless	optimized
T_{min}	5	5	°C	fixed
T_{max}	25	30	°C	fixed
VPD_{max}	1.5	1.5	kPa	fixed
VPD_{min}	3.5	4.0	kPa	fixed
f_{phena}	0.5	*	dimensionless	fixed
f_{phenb}	0.83	*	dimensionless	optimized
TT_{max}	600	*	°C days	fixed
TT_{end}	1400	*	°C days	fixed
coupled model: stomatal BBL model				
g_0	0.013	0.028	mol m^{-2} s^{-1}	derived from g_{st} measurements in the dark
a_1	5.1	5.5	dimensionless	optimized
Γ	50	45	ppm	derived from A_n/C_i curves
VPD_0	2.2	5.0	kPa	optimized
VPD_{tresh}	1.5	1.5	kPa	fixed
coupled model: photosynthesis model				
V_{m25}	160	162	µmol m^{-2} s^{-1}	optimized
J_{m25}	294	285	µmol m^{-2} s^{-1}	optimized
θ	0.1	0.1	dimensionless	optimized

3.2.2 Validation

Parameterization of both models was performed on a training set of data points; afterwards both models were validated against the test set of point measurements of g_{st} in ambient conditions and PAR response measurements. The coupled model was also evaluated in its ability to predict A_n. Plots of measured versus modelled g_{st} and A_n are shown in (Figure 11). The multiplicative model and the coupled model were able to explain 70% and 69% of the observed g_{st} variance for oilseed rape, respectively, as denoted by R^2. Both models slightly overestimated g_{st}, as indicated by the slopes of the linear regression of measured versus modelled g_{st}.

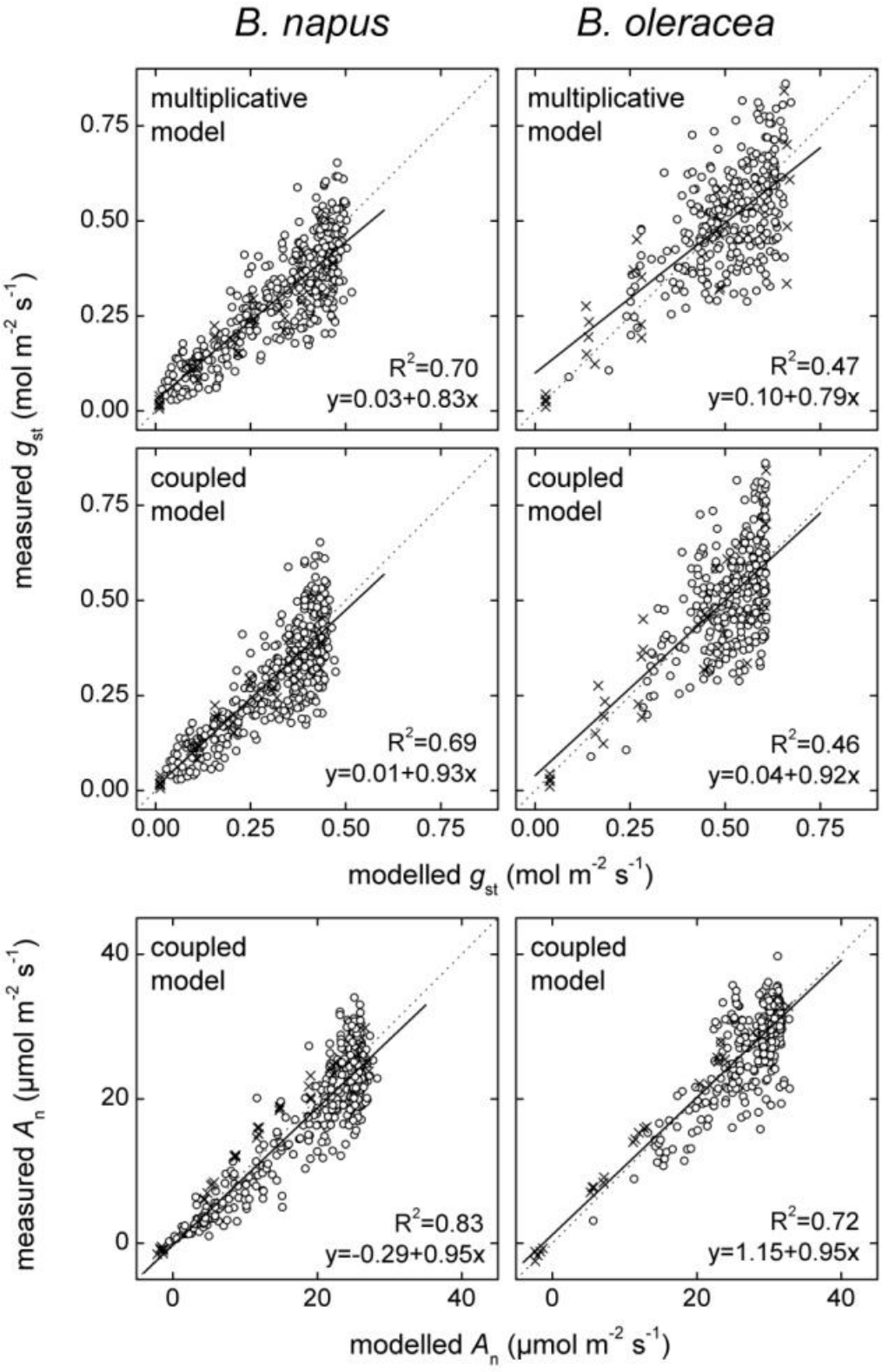


Figure 11: Validations results for oilseed rape (*B. napus*) (left graphs, n=404) and broccoli (*B. oleracea*) (right graphs, n=307). Measured versus modelled stomatal conductance to O_3 (g_{st}) and net photosynthesis (A_n). Open symbols are point measurements in ambient conditions and crossed symbols are PAR response measurements. Dotted lines depict the 1:1 relationship. Solid lines are the linear regression lines of measured versus modelled values. R^2=coefficient of determination (Op de Beeck *et al*, 2010).

In the case of broccoli (*B. oleracea*), the multiplicative model and the coupled model explained 47% and 46% of the observed g_{st} variance, respectively. Both models slightly overestimated g_{st}. The large intercept of the least squares regression for the multiplicative model indicates substantial model bias. As an aside, the ability of the coupled model to simulate A_n was relatively high. This was true for both species, as indicated by the regressions shown in Figure 11.

Both the multiplicative model and the coupled model were moderately successful in predicting g_{st} and gave better agreement with measured g_{st} for oilseed rape than for broccoli. We believe that the overall moderate performance of the models is less a result of a poor predictive capacity of the models themselves than of the nature of the data against which the models were validated. These data comprised measurements made on different leaves of different plants and, hence, contained a significant amount of physiological variability that the models could not account for. Also, it was also not guaranteed that the test set of gas exchange in ambient conditions only comprised measurements of steady-state g_{st}, even though the data set had been subjected to a selection with the goal to filter out measurements that did not approximate equilibrium. Since the models are steady-state models, deviations from steady-state g_{st} in the test data introduce prediction errors. From these it might wrongly be inferred that the models fail. In addition, part of the observed g_{st} variance might be due to endogenous plant factors that are not included in our environment-driven models. The better agreement of the models with measured g_{st} for oilseed rape than for broccoli is possibly due to the lack of data in the lower g_{st} range for broccoli (see Figure 11).

3.2.3 Discussion

In this study, the multiplicative model and the coupled model were equally successful in predicting g_{st}. Similar performance of both types of model has been shown before (Van Wijk *et al* 2000; Büker *et al* 2007) on grapevine, durum wheat, beech, birch and forest growth. Uddling *et al* (2005), however, tested both models against leaf-level measurements on silver birch and found the accuracy to be higher for the multiplicative model. Misson *et al* (2004), on the other hand found better results for the coupled model on ponderosa pine. In line with the general outcome of the above-mentioned studies, our results indicated an equal performance of the multiplicative model and the coupled model. In terms of predictive performance, the coupled model might thus provide a valid alternative to the multiplicative model for O_3 flux modelling. The evaluation of stomatal models for use in O_3 risk assessment should, obviously, not only be based on performance alone. Also other model characteristics such as input requirement have to be considered. Coupled models have a higher input requirement than multiplicative models, which - at first sight - favours the application of the latter over the former. For example, coupled models require A_n measurements for the parameterization of the photosynthesis submodel, an argument which has been put forward in disfavour of their use. With the advance of data acquisition methods and techniques, however, combined measurements of g_{st} and A_n rate at the leaf level (or of plant water and carbon exchange at the canopy level) have become common. Also, coupled models require substantially more input parameters to be tuned than multiplicative models. The coupled model applied in this study, for instance, contains twenty-five input parameters, while the multiplicative model used here only has twelve. Twenty of the twenty-five parameters are related to the photosynthesis submodel of Farquhar. A number of these parameters are claimed to be universal for C_3 plants (Bernacchi *et al*, 2001). They can be taken constant, which significantly reduces the number of photosynthesis parameters to be tuned. Alternatively, simpler photosynthesis models with less input parameters can be applied instead of the biochemical model of Farquhar, but their parameters might have less

physiological significance. Furthermore, the coupled model is able to simulate the effect of CO_2 concentration on g_{st}. To account for this effect the multiplicative model needs to be extended with a CO_2 function, leading to a further convergence of the number of input parameters of both models. The distance between the two models in terms of input parameters is thus not as large as it seems at first sight. Regardless performance and input requirement, coupled models have one major advantage over multiplicative models. Through the link between g_{st} and A_n, they offer the possibility to simulate effects on stomatal O_3 flux of factors that determine photosynthetic capacity such as O_3 damage, and effective stomatal O_3 fluxes by including a photosynthesis-related defense capacity (Massman *et al*, 2000). As pointed out by Büker *et al* (2007), such a mechanistic approach to the simulation of stomatal O_3 fluxes is particularly feasible on the local scale, where data needed for model parameterization are often readily available. Nevertheless, coupled models might also find their way to O_3 risk assessment on the regional scale in the long term.

3.2.4 Conclusion

The relative performance trends found in this study confirm the scarce literature available and support the coupled semi-empirical stomatal-photosynthesis model as a valid alternative to the multiplicative stomatal model for O_3 flux modelling, in terms of predictive performance. Both models allow the calculation of the so-called Phytotoxic Ozone Dose above a certain O_3 uptake rate threshold of x nmol m^{-2} s^{-1} (POD_x) to be used in O_3 dose-response functions and calculation of O_3 uptake over a wide range of climatic regions, as needed for the risk assessment of O_3 damage to vegetation.

3.3 Impact of elevated tropospheric O_3 on yield quantity of spring oilseed rape and broccoli (De Bock *et al*, 2011)

3.3.1 Calculation of O_3 exposure/dose indices and responses

The accumulated O_3 exposure over a threshold of 40 ppb (AOT40) was calculated as the sum of the differences between the hourly concentrations and 40 ppb for each daylight hour (global radiation > 50 W m^{-2}) when the concentration exceeded 40 ppb (Fuhrer and Achermann, 1994). The accumulated O_3 uptake is expressed as the phytotoxic O_3 dose ($POD_{x)}$, which is the cumulative uptake of O_3 per unit plant leaf area (PLA, mmol m^{-2}) based on hourly estimates of the O_3 uptake rate. O_3 uptake through the stomata was estimated using the previously described multiplicative stomatal conductance model (Jarvis, 1976) with a site-specific parameterisation for both *Brassica* species (Op de Beeck *et al*, 2010; see 3.2). An O_3 uptake rate threshold of 6 nmol m^{-2} s^{-1}, below which the O_3 uptake was not included, was incorporated in the calculations (POD_6). No threshold was used for POD_0. AOT40 and POD_x values were calculated for three different time frames during spring oilseed rape growth from 50% emergence until harvest: pre-anthesis exposure (from emergence until 50% of the plants were flowering), post-anthesis exposure (from 50% flowering until harvest) and total exposure (Table V). 50% flowering was recorded on 22 June 2007 (44 DAE), 6 June 2008 (46 DAE) and 24 May 2009 (43 DAE). For broccoli only total exposure from planting until harvest was considered.

For seed yield, oil yield, fresh marketable yield, oil percentage, 1000 seed weight and total dry weight the dose-response relationship was determined in a similar way (Table VI). For each individual growing season the reference value was extrapolated by linear regression as the yield to be expected at an AOT40 or POD_x equal to zero. Within each growing season the actual yield data were divided by the corresponding reference value to obtain the relative seed yield (RSY), relative oil yield (ROY), relative

fresh marketable weight (RFMW), relative oil percentage (RO%), relative 1000 seed weight (R1000SW) and relative total dry weight (RTDW). Linear regression analysis was performed to evaluate the relative yield parameter as a function of the O_3 exposure/uptake index, using data points from all three growing seasons. All regressions were forced through one at zero exposure/uptake level. Only little information is lost as the relative yield is supposed to be 100% at this point, because all yield parameters were divided by the yearly based reference value at zero exposure level. Slope and R^2 were calculated with Excel (Microsoft, 2007), level of significance of slope different from zero was calculated with SPSS (version 16.0). If the slope differed significantly, the critical O_3 level resulting in a yield reduction of 5% was determined.

Table V: Total O_3 exposure (AOT40) and uptake (POD_6) in the open top chambers from emergence until harvest of oilseed rape and broccoli for the growing seasons of 2007, 2008 and 2009.

	Oilseed rape			Broccoli		
Variable	2007	2008	2009	2007	2008	2009
AOT40 NF (ppm h)	0.71	2.11	1.51	0.38	0.74	1.28
AOT40 NF+ (ppm h)	5.66	10.74	10.95			
AOT40 NF++ (ppm h)	14.97	24.11	23.51	8.94	12.85	15.21
POD_6 NF (mmol m^{-2})	8.22	13.59	17.10	7.27	8.22	16.94
POD_6 NF+ (mmol m^{-2})	20.06	23.53	29.13			
POD_6 NF++ (mmol m^{-2})	31.29	38.08	43.72	24.37	28.89	40.14

NF: Non Filtered air: NF+, NF++: Non Filtered air with additional O_3; AOT40 accumulated ozone exposure over a threshold of 40 ppb; POD_6: Phytotoxic Ozone Dose over a threshold of 6 nmol m^{-2} s^{-1}.

3.3.2 O_3 dose-response functions for yield parameters

RSY of spring oilseed rape cv. Ability showed a significant decline in response to increasing AOT40 and POD_6 accumulated from 50% emergence until harvest (Figure 12). The highest O_3 values represent the NF++ treatment where 40 ppb of O_3 was added to the ambient level during 8 daylight hours. The corresponding AOT40 values over 20 ppm h led to a seed reduction of approximately 35% compared to an O_3 free environment and 30% compared to current O_3 levels (Figure 12 a). As such, seed yield of spring oilseed rape was significantly reduced by moderately elevated tropospheric O_3. The 8 hr average O_3 concentrations of NF and NF++ treatments are in the range defined for respectively current and future (year 2100) concentrations by Feng and Kobayashi (2009). However, since these projections are based on predictive studies with air-chemistry models (Meehl *et al*, 2007), there remains some uncertainty whether a 40 ppb increase is a realistic forecast of future O_3 concentration to be reached in the next 100 years (Oltmans *et al*, 2006). Some projections suggest an even greater increase to 80 ppb by 2100 (Fiscus *et al*, 2005). It must also be stressed that the calculated accumulated O_3 doses are very dependent on the exceedance of the O_3 threshold level and the light intensity, therefore the correlation with 8 hr averages is not always straightforward.

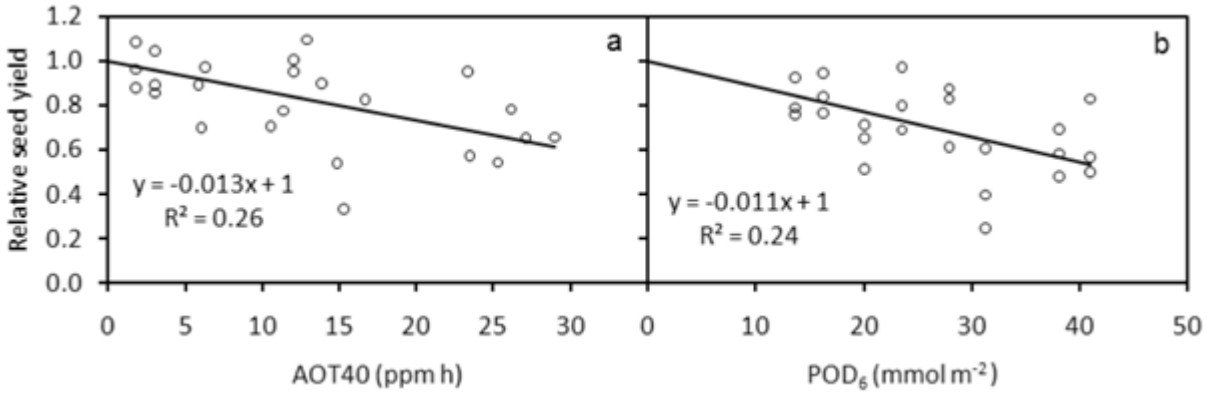


Figure 12: Correlation between relative seed yield and AOT40 (a) or POD_6 (b) for spring oilseed rape, calculated from 50% emergence until harvest.

RSY did not show a significantly higher correlation with the modelled stomatal O_3 uptake (POD_6) in comparison to the O_3 exposure (AOT40) accumulated from 50% emergence until harvest (Figure 12). This is rather surprising since it is expected that the plant's response is more tightly correlated with the flux-based POD_6 O_3 dose index, because it accounts for variations in environmental conditions that influence the actual stomatal O_3 uptake (Pleijel *et al*, 2004). It must be emphasised, however, that these data result from one site only, with a limited range of climatic variation (e.g. no soil water deficit). The flux approach will mainly prove its relevance under a wide range of climatic conditions over different locations e.g. from Southern to Northern Europe (Pleijel *et al*, 2007).

RSY was not significantly correlated to POD_0 for which, in contrast to POD_6, no threshold level for O_3 uptake is taken into account (Table VI). This may indicate that spring oilseed rape has the potential to neutralise part of the O_3 that is taken up by the stomata. Consequently, for the definition of a reliable critical O_3 dose it is necessary to include a threshold flux under which the O_3 uptake is not included in the total accumulated dose. Our results confirm that the value of 6 nmol s^{-1} m^{-2} PLA, as recommended for agricultural crops (Mills, 2004), can indeed be applied for spring oilseed rape. Based on our data the critical O_3 exposure level to prevent 5% seed yield is 3.7 ppm h AOT40 from emergence until harvest (Table VI). This implies that the suggested critical level of 3 ppm h for agricultural crops (Mills, 2004) will also protect spring oilseed rape. Furthermore, this critical level categorises spring oilseed rape as a rather O_3 sensitive crop such as turnip and onion (Mills *et al*, 2007). Nevertheless, winter oilseed rape - which is a different, but closely related genotype - seems less sensitive to O_3, with a critical level of 8.9 ppm h (Mills *et al*, 2007) based on previously published data (Ollerenshaw *et al*, 1999). ROY reduction was even more pronounced in comparison to RSY reduction (Table VI). This is due to an additional decrease in RO% (Tabel VI), possibly caused by a change in carbon partitioning due to increased allocation to leaf injury repair (Fiscus *et al*, 2005). Consequently, the critical AOT40 level to prevent a loss of oil production is lower compared to seed yield. The value of 3.2 ppm h approaches even closer the suggested critical level of 3 ppm h (Mills, 2004). The difference in correlation between linear regressions based on AOT40 and POD_6 was negligible (Table VI).

Table VI: Regression analysis of yield parameters for oilseed rape in correlation to AOT40 and PODx. Reference values refer to values obtained by linear regression at AOT40 or PODx = 0. The slope is given for y = ax + 1; *** corresponds to 0.001 significance level for the slope differing from 0.

Period (Variable)	Index	Reference value (2007/2008/2009)	Slope	R^2		Critical Level
Pre-anthesis	AOT40	279/398/371	-0.038	0.32	***	1.31 ppm h
(RSY)	POD_0	515/539/489	-0.027	0.36	***	1.88 mmol m^{-2}
	POD_6	357/428/404	-0.037	0.44	***	1.36 mmol m^{-2}
Post-anthesis	AOT40	275/376/379	-0.023	0.25	***	2.13 ppm h
(RSY)	POD_0	521/498/513	-0.012	-0.25	ns	
	POD_6	373/414/431	-0.015	0.03	ns	
Emergence-harvest	AOT40	278/369/369	-0.013	0.26	***	3.73 ppm h
	POD_0	518/511/507	-0.008	-0.01	ns	
(RSY)	POD_6	367/419/424	-0.011	0.24	***	4.36 mmol m^{-2}
Emergence-harvest	AOT40	42.8/43.9/43.6	-0.003	0.25	***	15.82 ppm h
	POD_0	53.4/47.8/44.3	-0.002	-0.08	ns	
(RO%)	POD_6	46.8/45.4/43.9	-0.003	0.11	***	17.62 mmol m^{-2}
Emergence-harvest	AOT40	117/161/163	-0.016	0.29	***	3.23 ppm h
	POD_0	230/230/236	-0.009	0.03	ns	
(ROY)	POD_6	158/185/192	-0.012	0.28	***	3.90 mmol m^{-2}
Emergence-harvest	AOT40	2.29/3.47/3.76	-0.006	0.17	***	8.54 ppm h
	POD_0	3.24/4.19/4.23	-0.005	-0.11	ns	
(R1000SW)	POD_6	2.66/3.73/3.94	-0.006	0.11	***	9.00 mmol m^{-2}

RSY: Relative Seed Yield; RO%: Relative oil percentage; ROY: Relative Oil Yield; R1000SW: Relative 1000 seed weight; AOT40 accumulated O_3 exposure over a threshold of 40 ppb; POD_6: Phytotoxic O_3 Dose over a threshold of 6 nmol m^{-2} s^{-1}; POD_0: Phytotoxic ozone dose without threshold; ns: not significant; Reference values are in g/m² for seed and oil yield, in % for oil percentage, in g for 1000 seed weight.

The reduction of RSY was most highly correlated with the O_3 exposure/dose accumulated between 50% emergence and anthesis, as compared to the other growth periods (Table IV). This is in contrast with previous results on potato and wheat (Pleijel *et al*, 1998; Pleijel *et al*, 2004), where O_3 exposure in the period from anthesis to harvest was considered more important. The highest R^2 value was obtained for POD_6 calculated over the pre-anthesis period. In support of these findings, R^2 for RSY correlated with both POD_6 and AOT40, was analysed for different time frames between emergence and harvest. POD_6 showed higher correlations with RSY compared to AOT40, but this is only true for time frames covering the first half of the the growing season. When the entire exposure period or the second half of the growing season is taken into account, both indices are lower and perform at a comparable level. A maximum value of 0.545 for R^2 is obtained if the POD_6 calculation is restricted from DAE 23 until 47 (Figure 13), which corresponds to a period of very fast vegetative growth until the onset of flowering (Al-Barzinjy *et al*, 2003). The high biomass accumulation in this early period is important for subsequent

seed development. This observation indicates that the use of a three month integration period for risk assessment of O_3 exposure may be too long, as was already suggested for wheat (Pleijel *et al*, 1998). The period during which plants are most sensitive to O_3 could be shorter, and is dependent on plant species and site. If the integration period is limited to the most sensitive growth stage, the reliability of the risk assessment for O_3 induced yield loss in spring oilseed rape will be improved.

The broccoli dose-response functions for RFMW and RTDW were not significantly correlated to the accumulated O_3 exposure or uptake and slope values of the regression approached zero (data not shown). Also on other cabbages like *Brassica campestris* (Chinese cabbage), O_3 had few effects on yield, although some physiological effects were noticed (Black *et al*, 2007). Yield effects on broccoli have been observed in response to other abiotic stress conditions such as higher root temperatures (Diaz-Perez, 2009) and low temperatures, especially when freezing occurred at the floral initiation stage (Tan *et al*, 1999). There might be several reasons why we did not find any O_3-induced yield effects for broccoli vegetables. First of all, the total O_3 exposure period for broccoli was much shorter compared to spring oilseed rape, which was exposed during its entire life cycle until seed set (although a small period proved more important). Broccoli was planted in the OTCs only after five weeks precultivation in the greenhouse and the vegetables were harvested before flowering. Eventhough the primary cause of the final yield effects may find its origin already before anthesis, as was shown here for spring oilseed rape, this does not imply that these effects are already measurable at such an early stage. Most effects of O_3 in plants occur at a later stage (Mullholland *et al*, 1998) and are often attributed to accelerated senescence (Vandermeiren *et al*, 2005). For broccoli no variation in integration period has been made, as the exposure period was too short.

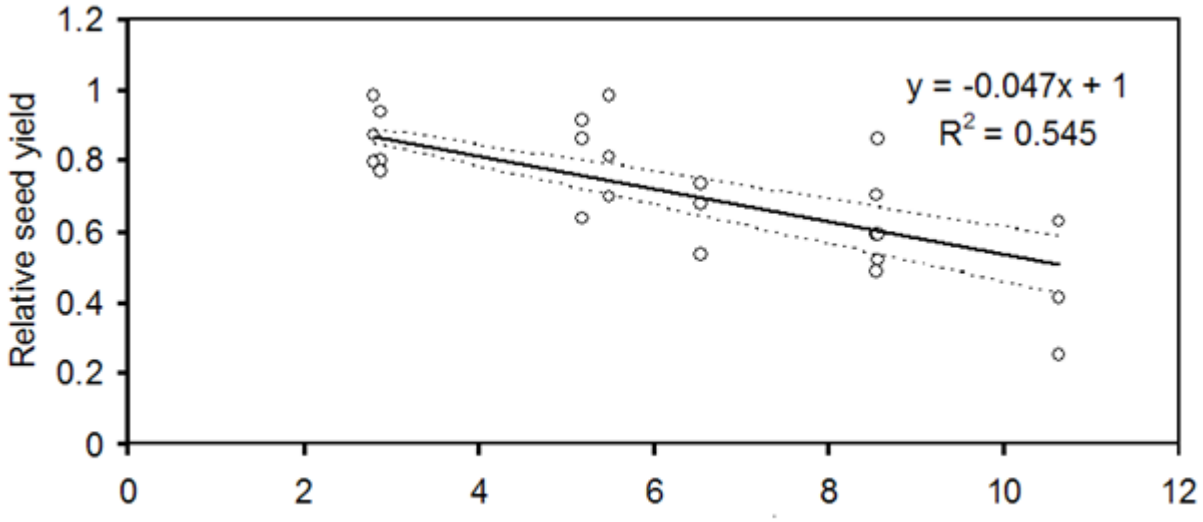


Figure 13: Correlation between POD_6 from 23 to 47 DAE and the relative seed yield. Dotted lines represent the 95% confidence interval.

3.3.3 Conclusions

Economic losses are expected for oilseed rape if O_3 concentrations continue to rise. In comparison to the current situation, seed yield losses of spring oilseed rape may be reduced by 30% within 100 years if future ambient 7 or 12 hr average O_3 concentrations increase to a range of 51 – 75 ppb, as predicted by Assessment Report Four (Meehl *et al*, 2007). Oil yield is even more affected due to an additional decrease of the oil percentage. Based on our data the critical AOT40 to prevent 5% seed or oil yield are

respectively 3.7 and 3.2 ppm h from emergence until harvest; the corresponding critical POD_6 values are 4.4 and 3.9 mmol m^{-2} (Table VI). This implies that the suggested critical level of 3 ppm h for agricultural crops (Mills, 2004) will also protect spring oilseed rape. Although there were no major differences in correlation between the AOT40 or POD_6 based regressions, the most significant O_3 yield response was obtained with the flux-based POD_6 accumulated during the limited period before anthesis, especially from 23 to 47 DAE ($R^2 = 0.55$). Moreover, the POD_6 dose response allows a more reliable extrapolation of O_3-induced yield losses over a wider range of climatic conditions. Based on our results, for broccoli no yield losses due to elevated O_3 are expected. This may be due to the short growth period and limited O_3 exposure but possibly the detrimental O_3 effects on marketable yield were not yet measurable because harvest occurs before flowering.

3.4 Impact of tropospheric O_3 on quality of oilseed rape and broccoli yield

(Vandermeiren *et al*, submitted)

O_3 induced changes in food and feed quality have been investigated in only a limited number of crops and most studies deal with carbohydrate and crude protein content. However, crop quality may also be affected by changes in secondary metabolism e.g. due to a diversion of available resources from growth to defence including increases in antioxidant scavenging systems within the tissue such as GSH, vitamin C and E (Iriti and Faoro, 2009). Despite several studies on the biochemical and molecular mechanisms of oxidative stress, only very little information exists on shifts of secondary metabolites in the marketable yield products (grains, tubers, fruits, vegetables). Such changes may however be crucial for industrial processing of the harvested products and for consumer's health. Therefore this project aimed to develop O_3 dose-response functions for a number of important quality traits of oilseed rape and broccoli. The calculation of these relationships is based on the method described in 3.3.1. with the exception that only AOT40 and POD_6 were included as independent x-factor, not POD_0.

3.4.1 O_3 effects on seed quality of oilseed rape

Oilseed rape is the third most important world source of vegetable oil (Lühs and Friedt, 1994). It can serve as a biofuel or a renewable resource for industrial applications. Moreover, the seeds are rich in linoleic and linolenic acids, essential polyunsaturated fatty acids and precursors of the Omega 6 and Omega 3 fatty acid families. They are of vital importance in mammalian nutrition, but since mammalian metabolism does not allow the build-up of the double bonds which these fatty acids contain, they must be supplied as fatty acids in the food. The presence of vitamin E in vegetable oil is important in relation to oil stability and nutritional labelling and thus also for possible health effects related to the consumption of oils (Gliszczynska-Swiglo and Sikorska, 2004). In rapeseed oil, vitamin E occurs as a mixture of two predominant forms, α-TOC and γ-TOC, which differ in their bioactivity and antioxidant properties. When the seeds are processed and de-oiled, the quality of the remaining rapeseed meal as feed supplement is not only dependant on its protein content. The GSL concentration is also important since these components may decrease digestibility and cause goitre or haemolytic anaemia if supplemented at excessive rates (Stoewsand, 1995).

Table VII summarises the results of the O_3 exposure/dose responses of quality parameters for oilseed rape. On the whole the significance of the linear regressions was comparable whether AOT40 or POD_6 was chosen as the independent x-value.

Table VII: Linear regression analysis of seed quality parameters for spring oilseed rape in correlation to AOT40 and POD_6 from emergence until harvest. The reference values at AOT40 and POD_6 = 0 have been extrapolated for each growing season. The slope is given for y = ax + 1. Significance of the slope: *p < 0.05; **p < 0.01; ***p < 0.001; ns p > 0.05. (+) and (-) denote a positive or negative quality effect

Parameter	Index	Reference			Slope*10^{-3}	R^2	Sign
		2007	2008	2009			
Total GSL (µmol g^{-1})	AOT40	6.75	5.20	12.1	-1.0	< 0.01	ns
	POD_6	6.44	5.37	12.7	-0.9	0.01	ns
Aliphatic GSL (µmol g^{-1})	AOT40	3.84	3.45	8.12	-2.3	0.02	ns
	POD_6	3.91	3.58	8.53	-2.2	0.08	ns
Indolic GSL (µmol g^{-1})	AOT40	2.91	1.75	3.94	0.5	< 0.01	ns
	POD_6	2.54	1.79	4.18	1.2	0.01	ns
Protein (% DW)	AOT40	24.4	24.1	24.7	2.1	0.32	(+)**
	POD_6	23.8	24.0	23.7	2.0	0.56	(+)***
Weight % saturated fatty acids	AOT40	8.53	6.90	6.86	1.9	0.15	(-)*
	POD_6	8.87	6.68	6.64	1.3	0.16	(-)*
Weight % mono-unsaturated fatty acids	AOT40	58.3	63.8	65.2	-1.1	0.17	(-)*
	POD_6	57.1	64.9	66.3	-0.7	0.16	(-)*
Oleic acid (%)	AOT40	56.3	62.0	63.5	-1.2	0.17	(-)*
	POD_6	55.1	63.2	64.7	-0.7	0.16	(-)*
Linoleic acid (%)	AOT40	23.0	19.2	18.6	3.0	0.30	(+)**
	POD_6	23.8	18.3	17.8	2.2	0.33	(+)**
Linolenic acid (%)	AOT40	9.51	9.66	8.62	0.6	0.05	ns
	POD_6	9.52	9.62	8.60	0.4	0.08	ns
α-TOC (mg 100 g^{-1})	AOT40	13.1	12.9	12.4	-0.1	< 0.01	ns
	POD_6	13.0	13.1	12.4	-0.1	< 0.01	ns
γ-TOC (mg 100 g^{-1})	AOT40	15.7	16.9	18.1	-3.1	0.36	(-)***
	POD_6	15.4	17.5	19.1	-2.3	0.45	(-)***
Total vitamin E (mg 100 g^{-1})	AOT40	29.2	30.1	30.9	-1.8	0.23	(-)**
	POD_6	28.9	30.9	31.9	-1.4	0.33	(-)**

DW: dry weight; GSL: glucosinolate; TOC: tocopherol

Oleic acid, a monounsaturated fatty acid, was by far the most abundant (approximately 60% of total fatty acids), followed by linoleic acid (20%) and linolenic acid (9%). These three fatty acids account for about 90% of the total fatty acid content. The relative percentage of linoleic acid was increased ($p < 0.01$) at the expense of oleic acid ($p < 0.05$). The percentage linolenic acid remained unchanged. Although the content of saturated fatty acids was very low (myristic < 0.05%, palmitic 4.6%, stearic 1.7%, arachidic 0.6%, behenic 0.4% and lignoceric acid 0.2%), their relative percentage increased at higher O_3 concentrations ($p < 0.05$) (Fig 14, Table VII). This was mainly due to the significant increase in palmitic acid (slope f(AOT40) = 0.0027, $R^2 = 0.22$, $p < 0.05$); slope f(POD_6) = 0.0019, $R^2 = 0.25$, $p < 0.01$). Other monounsaturated fatty acids besides oleic acid were palmitoleic, eicosenoic, eruric and

nervonic acid of which the total percentage remained below 2% and this was sligthly, though significantly increased by O_3 (slope f(AOT40) = 0.0016, R^2 = 0.22, $p < 0.05$); slope f(POD_6) = 0.0013, R^2 = 0.29, $p < 0.01$). However, this minor increase did not have an influence on the response of the total % monounsaturated fatty acids that was mainly determined by the decrease in oleic acid.

Vitamin E content of the seeds consisted mainly of α and γ-TOC; δ-TOC quantities were negligible. Vitamin E was significantly reduced ($p < 0.01$) at increasing O_3 concentrations. This was mainly due to the decrease in γ-TOC whereas α-TOC was not affected.

The protein concentration in seeds of this spring oilseed rape cultivar was significantly ($p < 0.01$) increased in response to higher O_3 exposure levels (Table VII). The total GSL content of the seeds was not significantly changed under increased O_3 exposure (Table VII). Neither was there a change in the group of aliphatic (with a side chain derived from methionine) or indolic GSLs (derived from tryptophan). The seeds mainly contained progoitrin (3.0 ± 0.27 µmol g^{-1}), gluconapin (1.6 ± 0.21 µmol g^{-1}) and 4OH-glucobrassicin (2.7 ± 0.20 µmol g^{-1}); the concentrations of glucobrassicanapin, glucobrassicin, gluconasturtiin and neo-glucobrassicin remained below 0.5 µmol g^{-1}.

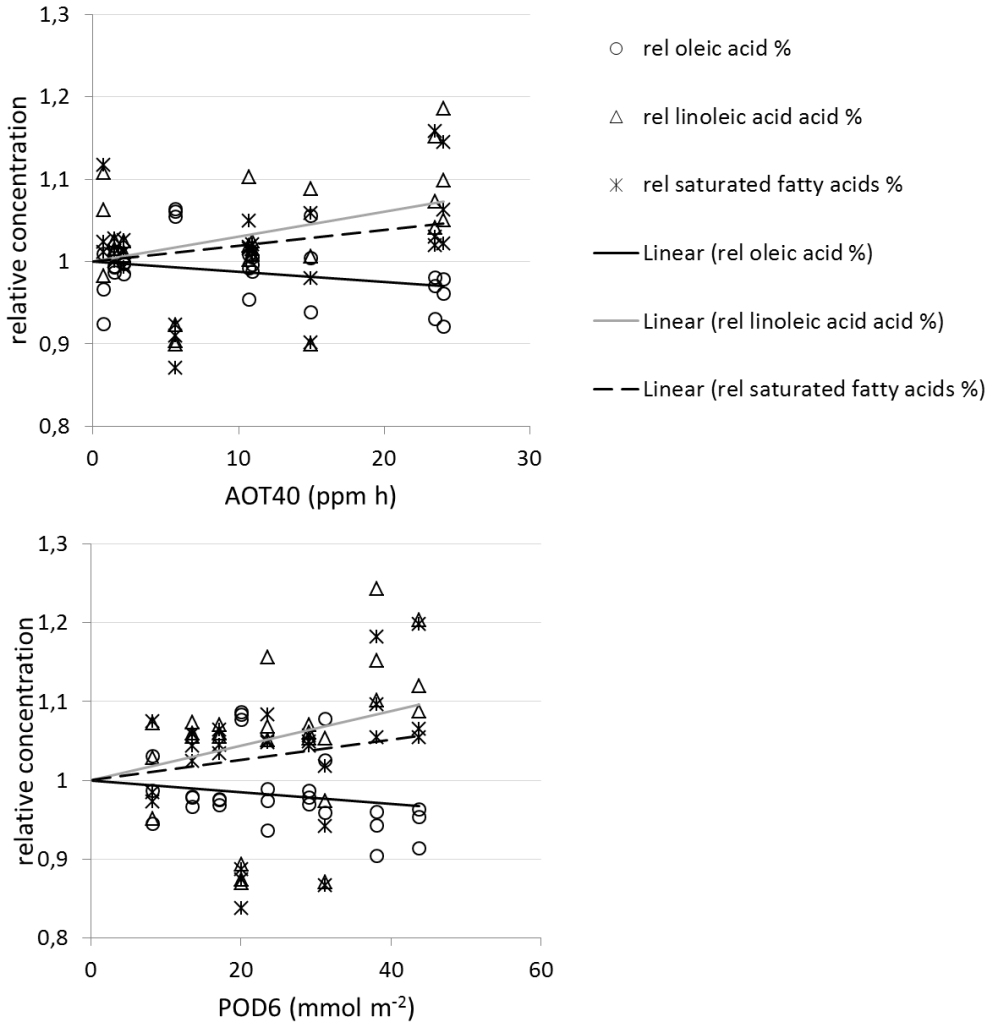


Figure 14: Linear regressions of the relative oleic, linoleic and saturated fatty acid % in seeds of spring oilseed rape in response to AOT40 and POD_6 accumulated from emergence until harvest,

3.4.2 O_3 effects on quality of broccoli vegetables

For the human diet, representatives of the *Brassicaceae* are of particular importance as vegetables. The protective effect of cruciferous vegetables against cancer has been suggested to be partly due to their high content of GSLs which distinguish them from other vegetables (van Poppel et al., 1999). There was a significant difference between the GSL concentrations over the different growing seasons (see reference values Table VIII), but without any O_3 x year interactions (ANOVA). In contrast to oilseed rape, the GSL content of broccoli heads was clearly influenced by O_3. The addition of 40 ppb O_3, 8 hrs per day, caused an increase of the aliphatic GSLs glucoiberin ($p = 0.05$) and glucoraphanin ($p = 0.08$) in the harvested vegetables, the latter being the most abundant GSL in broccoli (Fig 15). This did not, however, result in a significant increase of the total GSL content as the effect was counteracted by a decrease of the indolic GSLs glucobrassicin ($p < 0.01$) and neoglucobrassicin (ns) (Fig 15). As a consequence, there was a highly significant ($p < 0.001$) increase of the aliphatic/indolic GSL ratio from 0.97 to 1.72 in the NF++ versus the NF treatment (Fig 16). The linear regressions of the relative GSL concentrations as a function of AOT40 and POD_6 confirmed these O_3 effects: the relative aliphatic GSL concentration showed a strong tendency to increase in response to AOT40 ($p = 0.07$) and POD_6 ($p = 0.05$), whereas the indolic GSLs were significantly reduced ($p < 0.01$ resp $p < 0.001$) (Table VIII).

The protein content of broccoli vegetables was significantly increased in response to a higher O_3 exposure/uptake (Table VIII). With the exception of GSH, that showed a tendency to rise in response to O_3 (but only significantly for POD_6), none of the other antioxidants ASC and α-TOC were influenced by the O_3 treatment (Table VIII).

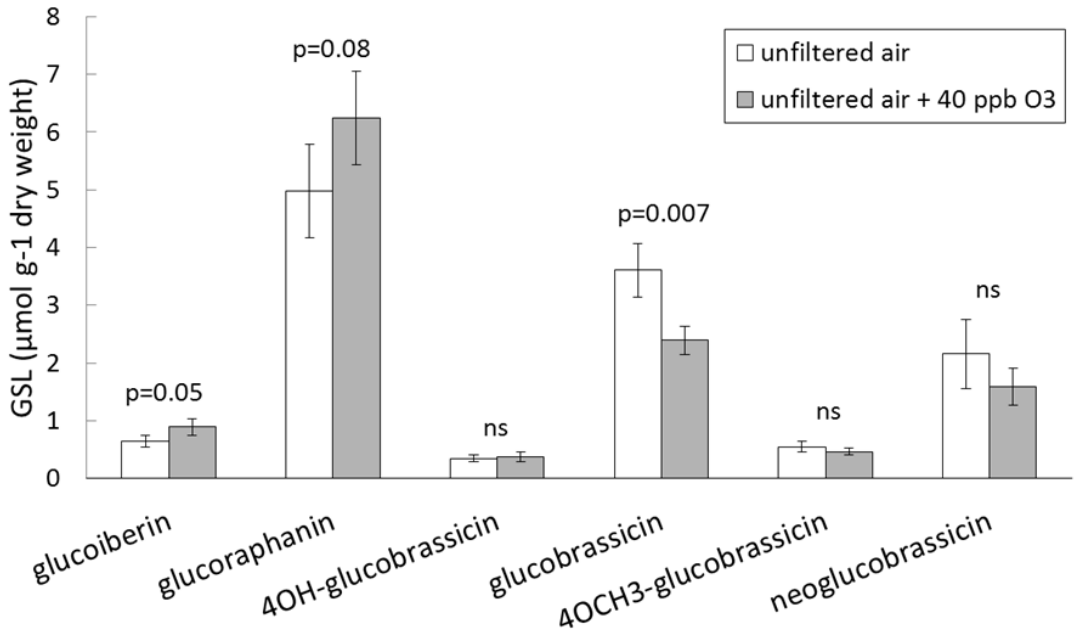


Figure 15: Effects of elevated O_3 on glucosinolate (GSL) composition and content of broccoli vegetables. Significance of the O_3 effect was determined by two-way ANOVA and is indicated on top of the bars.

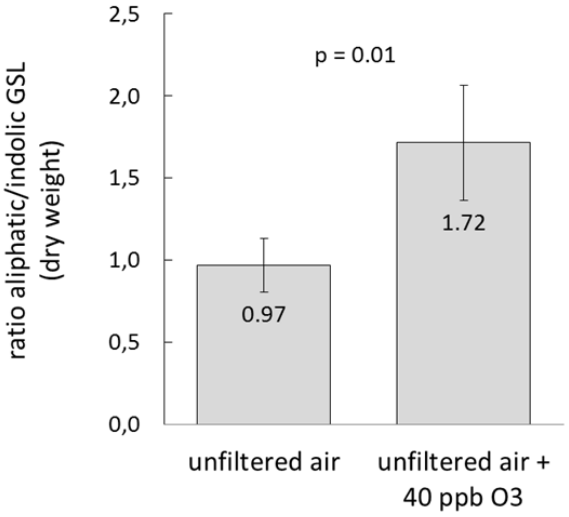


Figure 16: Ratio of aliphatic/indolic glucosinolates (GSL) in broccoli heads at harvest

Table VIII: Regression analysis of quality parameters for broccoli vegetables in correlation to AOT40 and POD_6 from planting until harvest. The reference values at AOT40 and POD_6 = 0 have been extrapolated for each growing season. The slope is given for y = ax + 1; *p < 0.05; **p < 0.01; ***p < 0.001; ns p > 0.05. (+) and (-) denote a positive or negative quality effect; for (?) see 3.4.3

Parameter	Index	Reference			Slope*10^{-3}	R^2	sign
		2007	2008	2009			
Total GSL	AOT40	7.76	11.6	17.9	0.5	< 0.01	ns
(µmol g^{-1} DW)	POD_6	6.04	12.2	20.0	2.4	0.01	ns
Aliphatic GSL	AOT40	4.34	3.29	9.02	25	0.18	ns (p=0.07)
(µmol g^{-1} DW)	POD_6	2.80	2.98	9.29	19	0.20	ns (p=0.05)
Indolic GSL	AOT40	3.21	8.30	8.89	-20	0.37	(?)**
(µmol g^{-1} DW)	POD_6	3.18	9.21	10.7	-9.8	0.48	(?)***
Aliph/indol GSL	AOT40	1.34	0.43	1.02	61	0.57	(+)***
	POD_6	0.81	0.31	0.69	50	0.66	(+)***
Protein (% DW)	AOT40	3.90	4.56	4.61	4.1	0.40	(+)**
	POD_6	3.84	4.47	4.48	2.3	0.60	(+)***
ASC total (µmol g^{-1} FW)	AOT40	6.02	5.11	5.92	-1.4	0.02	ns
	POD_6	5.98	5.10	6.14	-1.0	0.06	ns
GSH total (µmol g^{-1} FW)	AOT40	0.69	0.41	0.92	9.9	0.12	ns
	POD_6	0.74	0.40	0.72	9.1	0.29	(+)*
α- TOC (µmol g^{-1} FW)	AOT40	0.68	0.72	2.54	29	0.15	ns
	POD_6	0.67	0.47	2.60	19.5	0.17	ns

ASC: ascorbate; DW: dry weight; FW: fresh weight; GSL: glucosinolate; TOC: tocopherol

3.4.3 Conclusions

Our results provide clear evidence that not only yield effects, but also changes in food and feed quality of the harvested products are essential to determine the final economic costs and risks for human and animal health. Ozone induced oxidative stress caused a shift in both primary (proteins, fatty acids) and secondary metabolite concentrations (antioxidants, glucosinolates) of the marketable yield products of the investigated *Brassica* species. The significance of the responses was comparable whether they were expressed as a function of the accumulated O_3 concentrations AOT40 or modelled O_3 uptake POD_6. Depending on the crops, the specific quality parameter and the nature of the response, the effect of O_3 may be either beneficial or detrimental for the product quality.

With regard to the economic consequences of O_3 damage to crops, for oilseed rape the increase in protein content versus the reduction of the oil percentage needs to be taken into account in combination with the predicted yield losses (De Bock *et al*, 2011). Rapeseed oil is a valuable plant oil for human nutrition due to its high content of monounsaturated oil acids (mainly oleic acid), essential polyunsaturated fatty acids (linoleic and linolenic acid) in combination with a very low proportion of saturated fatty acids. O_3 has both negative as well as positive effects on these quality aspects since the relative proportion of oleic acid (18:1) is decreased whereas the linoleic acid % (18:2) and the weight % saturated fatty acids were increased (Fig 14). The cause of such shifts in relative fatty acid composition (e.g. by direct oxidation or altered lipid metabolism) is not known. The reduction of the vitamin E concentration of the seeds (Table VII) can be considered as a negative effect on the nutritional value of the oil. However, there was no significant effect on α-TOC, which is healthwise most important in vegetable oils (San Andrés *et al*, 2011). The decrease in γ-TOC is however qualitatively equally or maybe even more relevant to preserve the oxidative stability and increase their storage life (Kamal-Eldin and Appelqvist, 1996; Yanishlieva *et al*, 2002). Our spring oilseed rape cultivar Ability is a so-called double low variety which implies that the GSL level in the seeds is very low (less than 20 µmol g^{-1} seed dry matter). For ruminants, such low-GSL rapeseed meal can be used as the sole protein supplement without any apparent adverse effects on animal health (EFSA, 2008). O_3 exposure did not result in any significant changes in GSL content or composition of *Brassica napus* seeds; so no consequences are to be expected with regard to feed safety. These low concentrations however also implicate that statistically significant changes are more difficult to detect. Therefore it would be interesting to investigate this in *e.g*. winter cultivars of *Brassica napus* with a higher endogenous GSL content.

Despite the fact that elevated O_3 did not have an effect on the fresh weight of broccoli vegetables (De Bock *et al*, 2011; see also 3.3), their quality was undoubtedly influenced. A reason for the different response among the GSL groups (increase in aliphatic versus decrease of indolic GSLs) could be that the various enzymes involved in each GSL' synthesis are affected differently. The conversion of tryptophane to indolic GSLs is catalysed by peroxidases (Starzynska *et al*, 2003) whereas the level of methionine-derived GSLs is mainly determined by the initial entry of methionine into the pathway. The concentration and composition of the GSLs also changes during plant development. In broccoli e.g., the highest content of glucoraphanin occurs at the mature head stage and then declines as flowering is initiated (Rangkadilok *et al*, 2002). Consequently, the observed effect of O_3 on GSLs may also be due to its influence on changes in the rate of plant development. A reduction of the photosynthetic capacity, which is a common response to O_3 (Vandermeiren *et al*, 2005), may also have an impact on the plant's carbon content and its GSL levels. The consequences of these changes in GSL content and ratios on the health promoting properties of broccoli vegetables need to be carefully considered. The prevailing mechanism for the anticarcinogenic activity of these compounds has been considered to be the

induction of mammalian detoxication and antioxidant (phase II) enzyme activity by the GSL hydrolysis products, isothiocyanates. Sulphoraphane, the isothiocyanate from glucoraphanin, is still considered the most potent inducer of phase II enzyme (Verkerk *et al*, 2009). Therefore broccoli has been deliberately breeded and selected for higher levels of glucoiberin and glucoraphanin (Faulkner *et al*, 1998; Mithen *et al*, 2003). Indole-3-carbinol, a hydrolysis product from indolic GSLs, on the other hand, is considered to both inhibit and promote carcinogenesis (Stoner *et al*, 2002) and appears responsible for the modulation of estrogen receptor activity (Rahman and Sarkar, 2002). Such findings complicate conclusions in relation to the health effects of GSLs for humans. But overall the increase of the ratio of alphatic/indolic GSLs in response to O_3 may be considered beneficial.

3.5 Are the yield effects related to changes in leaf physiology and crop growth?

(De Bock *et al*, submitted)

From this three year study we previously reported clear yield reductions for oilseed rape yield that were most significantly correlated with the O_3 exposure during vegetative growth (see 3.3). However, broccoli yield was not affected (De Bock *et al*, 2011 and 3.3). The aim of this section is to assess the impact of the applied O_3 treatments on the growth and physiological performance of both crops to obtain information on the possible mechanism of these differential yield effects. Yield losses, and subsequent economic losses, can be caused by a reduction in photosynthetic biomass production, with possible changes in assimilate partitioning, or by an increase in senescence, which may lead to a loss in biomass by shortening of the active accumulation period (Ding *et al*, 2007). Photosynthetic activity is closely related to chlorophyll a fluorescence emission; variation of the chlorophyll fluorescence yield or heat dissipation provides indirect information on the efficiency of the photosynthetic apparatus. Therefore net photosynthetic rate (A_{sat}), stomatal conductance (g_{st}), actual quantum yield of photochemistry ($\Phi_{PSII} = F'_v/F'_m$), maximum quantum yield of Photosystem II (F_v/F_m), vitality performance index (PI) and the chlorophyll content of the fully developed upper canopy leaves were monitored on a weekly basis from emergence until crop harvest. For certain measurements also a labelled lower canopy leaf was monitored during its further development. Leaf area index (LAI) was used to quantify the evolution of crop growth non-destructively. For further details on methodology see 2.2.

3.5.1 Calculations and statistics

To allow comparison of all these parameters over different growing seasons, thermal time (TT) above 0°C was calculated as the sum of the mean daily temperatures over time, starting at 50% emergence for oilseed rape and after planting for broccoli. To evaluate effects of enhanced O_3 (NF+, NF++) on leaf physiology and crop growth each parameter value in the individual OTCs of the elevated O_3 treatments (NF+ or NF++) was divided by the mean value of this parameter under NF conditions, measured at the same date. For oilseed rape a distinction was made between measurements before and after the onset of flowering at approximately 730°C days. Slope and R^2 were calculated after linear regression with Excel (Microsoft, 2007), level of significance of slope different from zero was calculated with SPSS (version 16.0). Significant differences between NF and the elevated O_3 treatments for all yield data at harvest and physiological parameters determined in lower canopy broccoli leaves were calculated using a two sample t test with Excel (Microsoft, 2007).

3.5.2 Influence of increasing O_3 on crop growth and leaf physiology of spring oilseed rape

In unfiltered OTCs a decline of LAI of *Brassica napus* crop set in shortly after the onset of flowering, around thermal time 750°C days (Figure 17 a). At the same time A_{sat} and g_{st} of the upper canopy leaves started to decrease (Figure 17 d, g).

Stomatal gas exchange and chlorophyll a fluorescence did not show any significant reduction in the NF+ treatment (Figure 17 e, h; Figure 18 b, e, h). In the most elevated O_3 treatment however A_{sat}, g_{st}, F'_v/F'_m, F_v/F_m and PI of the upper canopy leaves were all significantly reduced in comparison to ambient O_3, but only after maximum leaf area (MLA) was obtained, which coincides with the time that 50% of the plants are flowering (Figure 17 c, f, i; Figure 18 c, f, i). This is also the moment when crop senescence sets in: the plants do not produce any new leaves and lower canopy leaves start to drop (Figure 17 a). The plants start to translocate assimilates and energy to reproduction, leaving less energy for defence strategies against stressors such as ozone (Black *et al*, 2000). From this point in time, the measurements on the upper canopy leaves reflect the gradual leaf senescence, whereas before MLA, all physiological measurements are performed on young healthy leaves that have just reached full development. We may therefore conclude that the O_3 induced decrease of the physiological performance of the upper canopy leaves of oilseed rape is a reflection of increased senescence. The more pronounced decrease of LAI and chlorophyll content in the elevated O_3 treatments, even in NF+, is also a clear indication of earlier crop senescence (Figure 17 b, Figure 19 b). Nevertheless, linear regression analysis of the O_3 dose- yield response by De Bock *et al* (2011) (see 3.3.2) suggested that oilseed yield was most significantly correlated with O_3 uptake before flowering. This implies that, although the impact of O_3 canopy development and physiological performance only becomes measurable after MLA, these effects are already induced during earlier crop growth. It is obvious that the O_3 induced increased senescence that set in on the upper canopy leaves after flowering also occurred in the lower canopy, but already at an earlier stage (Figure 19 d, e). Consequently, the total CO_2 assimilation and biomass accumulation of the entire canopy will be reduced due to an earlier decrease of the photosynthetic capacity of each individual leaf, and this will lead to the final yield reduction. The decrease of A_{sat} was more pronounced compared to g_{st} (Figure 17 f,i), indicating that the decreased photosynthetic activity was not only a consequence of reduced CO_2 uptake due to stomatal closure. This is confirmed by the decrease in PI, F_v/F_m and F_v'/F_m', which indicates a reduced efficiency of the photosynthetic apparatus through the reduction of open PSII reaction centres (Bose, 1982; Nussbaum *et al*, 2001).

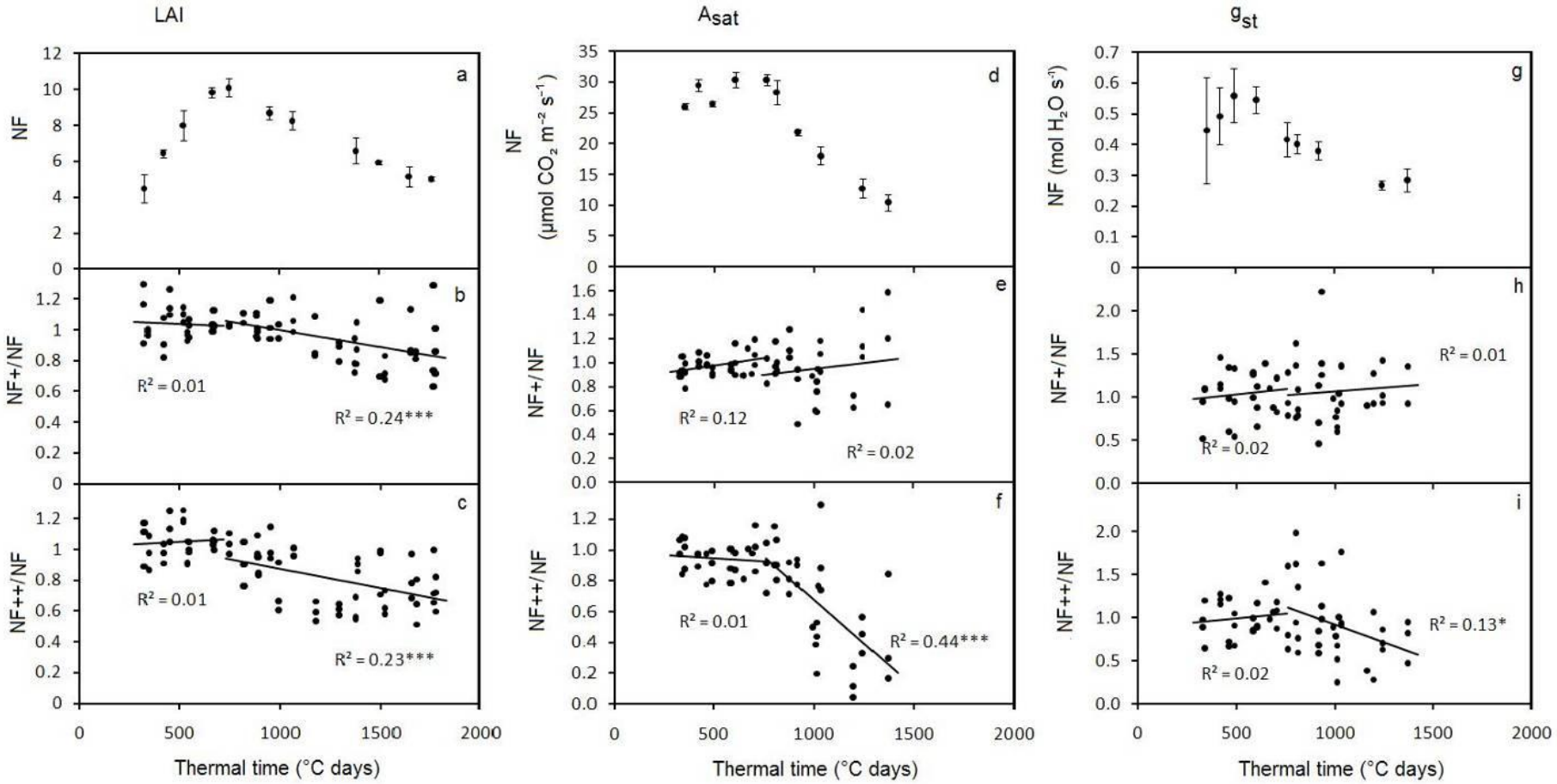


Figure 17: LAI and gas exchange in upper canopy leaves of oilseed rape: (a) evolution of leaf area index (LAI), (d) net CO_2 assimilation rate at saturating photosynthetic active radiation (A_{sat}) and (g) stomatal conductance (g_{st}) in function of thermal time for the NF treatment in 2009. The O_3 effect is expressed as the ratio's of NF+ and NF++ over NF in 2007, 2008 and 2009 for LAI (b,c), A_{sat} (e,f) g_{st} (h,i)) in function of thermal time. NF: Non Filtered air: NF+, NF++: Non Filtered air with additional O_3.

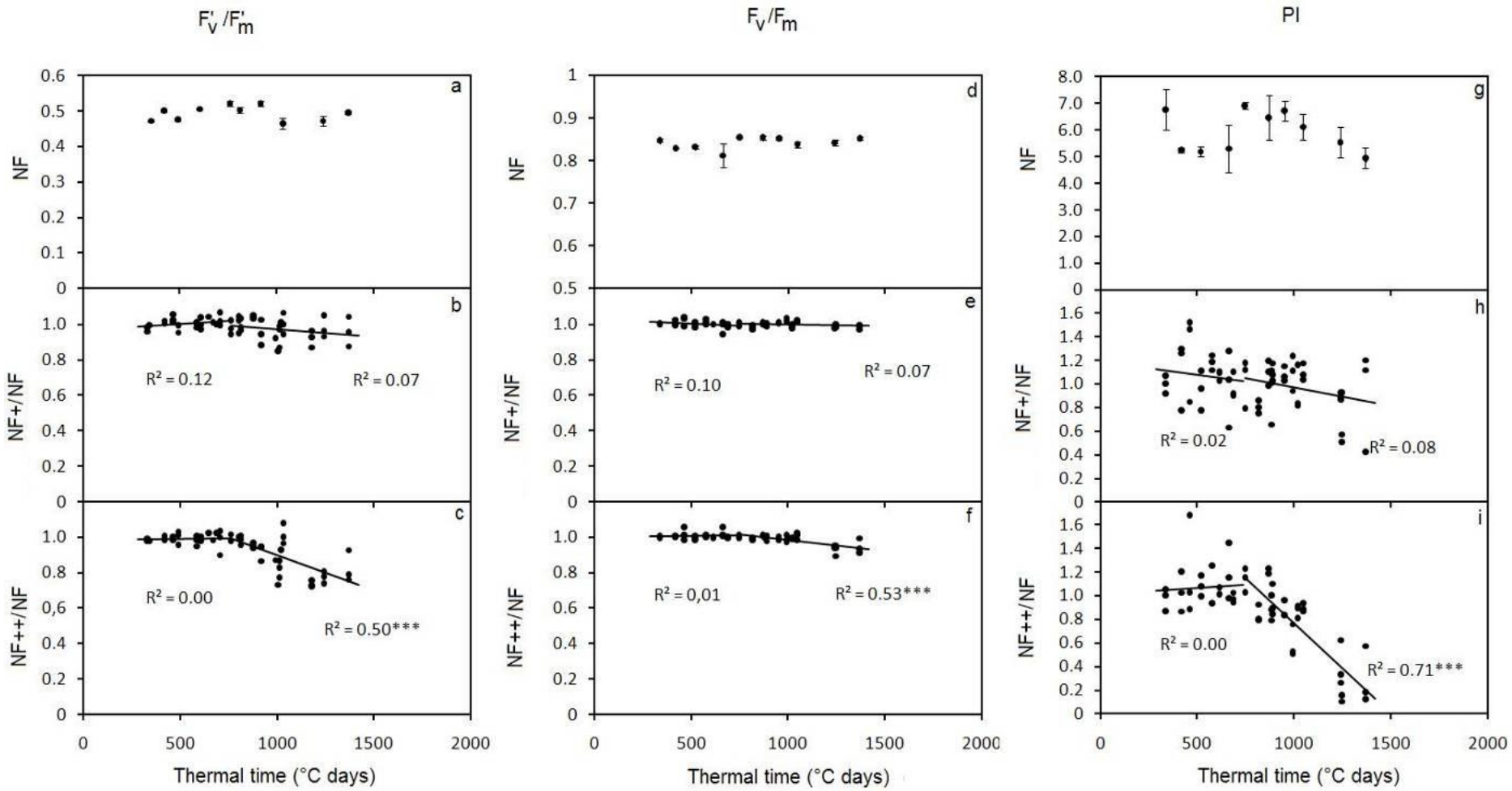


Figure 18: Chlorophyll a fluorescence in upper canopy leaves of oilseed rape: (a) evolution of the actual quantum efficiency of photosystem II (F'_v/F'_m), (d) the maximum quantum efficiency of photosystem II (F_v/F_m) and (g) the performance index (PI) in function of thermal time for the NF treatment in 2009, The O_3 effect is expressed as the ratio's of NF+ and NF++ over NF in 2007, 2008 and 2009 for F'_v/F'_m (b,c) , F_v/F_m (e,f) and PI (h,i) in function of thermal time. NF: Non Filtered air: NF+, NF++: Non Filtered air with additional O_3.

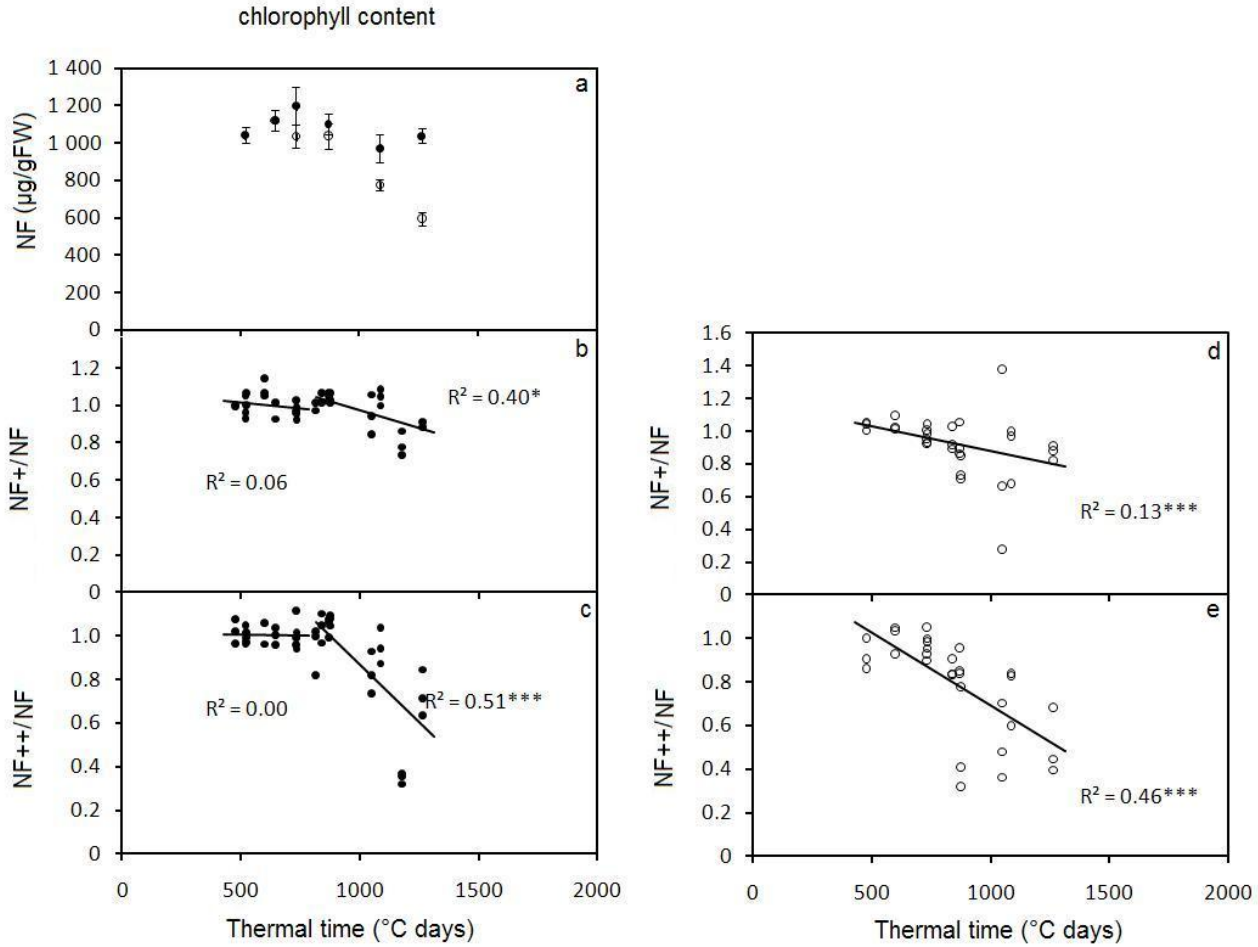


Figure 19: Chlorophyll content in upper and lower canopy leaves of oilseed rape in NF in 2009 (a). The O_3 effect is expressed by the relative chlorophyll content for NF+ (b, d) and NF++ (c, e) versus NF in upper (b, c) and lower (d, e) canopy leaves for 2007, 2008 and 2009 in function of thermal time. NF: Non Filtered air: NF+, NF++: Non Filtered air with additional O_3.

3.5.3 Influence of increasing O_3 on crop growth and leaf physiology of broccoli

In broccoli on the contrary, the exposure to elevated O_3 from planting to harvest did not cause a difference in fresh marketable yield nor in total aboveground dry weight (De Bock *et al*, see 3.3.2). This corresponds with the absence of O_3 induced changes on LAI and on the physiological performance of the upper canopy leaves: neither A_{sat}, g_{st}, PI, F_v/F_m, F_v'/F_m' or chlorophyll content showed any decrease in response elevated O_3 exposure (Figure 20 b,d,f, Figure 21 b,d,f). Nevertheless, as for senescing leaves of oilseed rape, the older lower canopy leaves of broccoli showed a significant reduction of A_{sat}, F'_v/F'_m, F_v/F_m, PI and chlorophyll content (Table IX).

Broccoli is harvested shortly after the end of the vegetative stage: flower buds were formed, but no flowers appeared on the broccoli plants yet. At this time, the plants are not yet losing any leaves (Figure 20 a) and upper canopy leaves have just reached full maturity. Nevertheless, the reduced chlorophyll content of the lower canopy leaves as registered in NF at that time (data not shown) indicates that senescence gradually sets in. This is again a confirmation that the expression of O_3 symptoms at the physiological level is highly dependent on leaf age. Moreover, total accumulated O_3 uptake is higher for these older leaves due to prolonged exposure. Apparently, in broccoli, the photosynthetic activity of the upper canopy leaves was sufficient to compensate for the loss in CO_2 assimilation in the older leaves so that the elevated O_3 exposure did not lead to a decrease of biomass production at the time of harvest. In addition, a realistic, but not extremely high O_3 dose was used in this research and the total exposure period was much shorter for broccoli compared to oilseed rape resulting in a lower accumulated O_3 uptake.

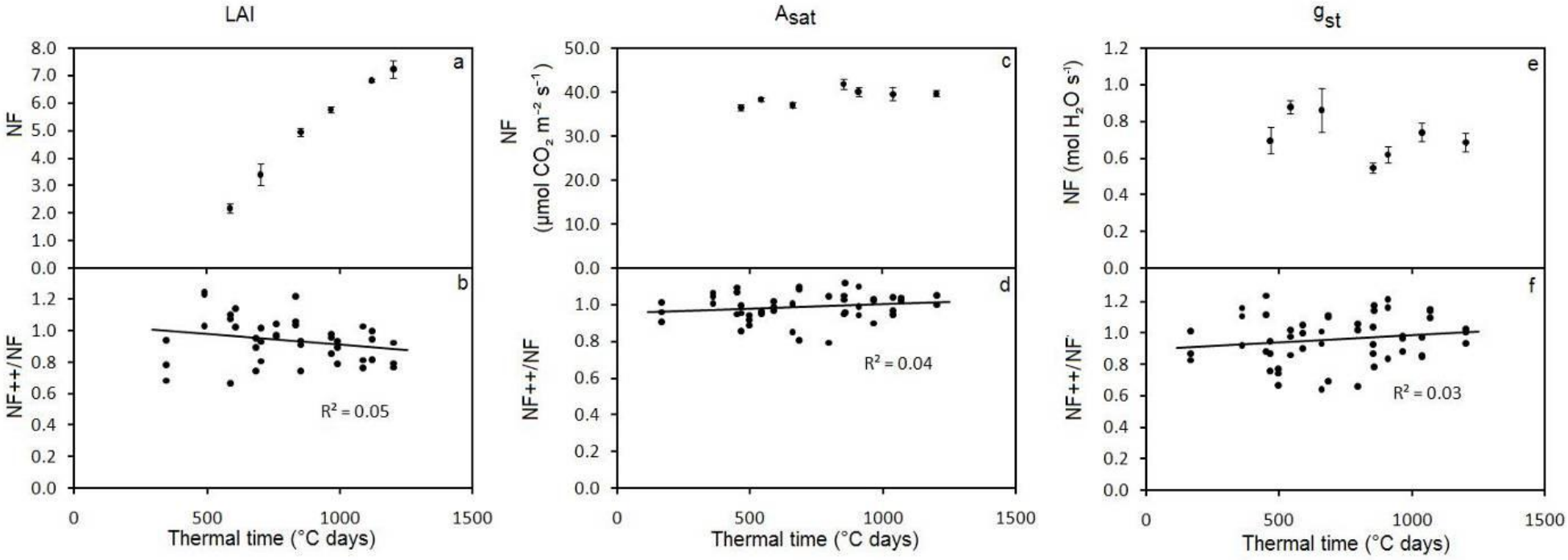


Figure 20: LAI and gas exchange in upper canopy leaves of broccoli: (a) evolution of leaf area index (LAI) , (c) net CO_2 assimilation rate at saturating photosynthetic active radiation (A_{sat}) and (e) stomatal conductance (g_{st}) in function of thermal time for the NF treatment in 2009. The O_3 effect is expressed as the ratio of NF++ over NF in 2007, 2008 and 2009 for LAI (b), A_{sat} (d) g_{st} (f) in function of thermal time. NF: Non Filtered air: NF++: Non Filtered air + 40 ppb O_3.

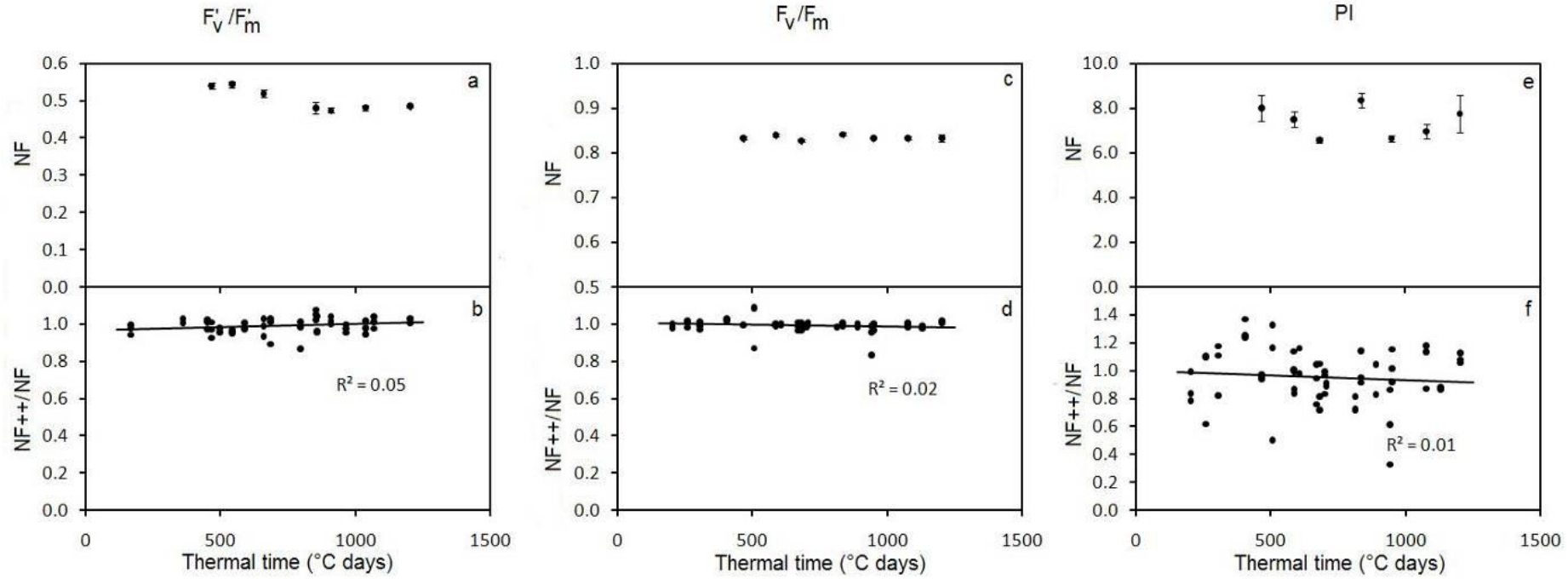


Figure 21: Chlorophyll a fluorescence in upper canopy leaves of broccoli: (a) evolution of the actual quantum efficiency of photosystem II (F'_v/F'_m) , (c) the maximum quantum efficiency of photosystem II (F_v/F_m) and (e) the performance index (PI) in function of thermal time for the NF treatment in 2009, The O_3 effect is expressed as the ratio of NF++ over NF in 2007, 2008 and 2009 for F'_v/F'_m (b) , F_v/F_m (d) and PI (f) in function of thermal time. NF: Non Filtered air: NF++: Non Filtered air + 40 ppb O_3

Table IX: Mean values (ste) of A_{sat}, g_{st}, F'_v/F'_m, F_v/F_m, PI and chlorophyll content in older broccoli leaves measured in NF and NF++ on July 30th 2008. Significance of the O_3 effect: ns: p>0.05, *: p<0.05, **: p<0.01)

	NF	NF++	significance
A_{sat} (µmol CO_2 m^{-2} s^{-1})	14.82 (3.09)	2.49 (1.55)	*
g_{st} (mol H_2O m^{-2} s^{-1})	0.292 (0.115)	0.059 (0.019)	ns
F'_v/F'_m	0.482 (0.008)	0.334 (0.084)	*
F_v/F_m	0.842 (0.003)	0.804 (0.016)	*
PI	6.26 (0.50)	3.83 (0.21)	**
[Chlorophyll] µg/gFW	989.0 (225.3)	420.4 (70.4)	*

3.5.4 Conclusions

It is concluded that the measured changes in canopy development and physiological performance of the upper canopy leaves of both plant species are in accordance with the observed yield effects. The reduction of the seed yield *of Brassica napus* cv Ability as a consequence of elevated O_3 exposure can be caused by the more rapid decrease in photosynthetic performance of the leaves due to earlier senescence. The absence of any biomass or yield effects for broccoli may be due to the fact that the CO_2 assimilation in the upper canopy leaves was not yet affected by the time of harvest which takes place at a much earlier growth stage, before flowering and MLA.

3.6 Ozone induced changes in leaf metabolism of oilseed rape and broccoli

3.6.1 Chronic O_3 exposure influences antioxidative defence mechanisms

When O_3 is taken up into the leaf, the extent of damage is highly dependent on the antioxidative defence capacity of the plant. O_3 causes the formation of reactive oxygen species (ROS) in the form of superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet-}$). It has been proven that sites where accumulation of H_2O_2 occurs correlate well with occurance of leaf lessions (Pellinen *et al*, 1999, Wohlgemuth *et al*, 2002). It is important for plants to effectively scavenge ROS to prevent damage either by enzymatic and/or by non-enzymatic mechanisms (Castagna and Ranieri, 2009). Non-enzymatic antioxidant defence strategies include ASC, GSH and TOC. α-TOC is a lipid soluble compound, exlusively synthesized in plants or other organisms with photosynthetic activity. This non-enzymatic antioxidant also plays an important role in protecting PS II against oxidative damage due to ROS, formed in the chloroplast as a by-product of photosynthesis (Golan *et al*, 2006). A rise in TOC concentrations can provide protection from oxidative damage by avoiding lipid peroxidation (Munné-Bosch, 2005; Maeda and DellaPenna, 2007). ASC has been shown to be the most abundant metabolite influencing the redox capacity in plants (Noctor and Foyer, 1998) and is therefore an important indicator for the total water soluble antioxidative capacity (TAC). The ASC content in plants can be regulated by the ratio of synthesis and degradation; furthermore oxidized ASC, like monodehydroascorbate free radical (MDHA•) or dehydroascorbate (DHA), can also be recycled through an enzymatic reduction involving GSH or nicotinamide adenine dinucleotide phosphate (NADPH), where DHA is reduced by oxidation of GSH, catalyzed by DHA reductase (DHAR). MDHA• can be reduced by monodehydroascorbate reductase (MDHAR) with an oxidation of NADPH (Asada, 1999; Foyer and Noctor, 2009). ASC synthesis is catalyzed through a series of reactions with guanosine diphosphate

(GDP)-mannose pyrophosphorylase, GDP-mannose-3,5-epimerase, L-galactono-1,4-lactone dehydrogenase, L-galactose dehydrogenase, L-galactose-1-phosphate phosphatase and L-galactose guanyltransferase (Smirnoff and Wheeler, 2000). GSH is considered the most abundant pool of reduced sulphur, which is not incorporated in proteins (Kunert and Foyer, 1993; Noctor *et al*, 1998). GSH synthesis is catalyzed by two enzymes: glutamate-cysteine ligase and glutathione synthetase (Meister, 1988). Oxidized GSH can be reduced by GSH reductase (GR) at the expense of NADPH (Noctor *et al*, 1998).In plants different enzymes are directly involved in catalyzing the reduction of ROS. Ascorbate peroxidase (APX) rapidly converts H_2O_2 into H_2O by oxidizing ASC. ASC also reduces H_2O_2 in a non-enzymatic way. Complementary to APX, superoxide dismutase (SOD) plays an important role in protection against oxidative damage. It neutralizes $O_2^{\bullet-}$ by oxidizing the metal core of the enzyme, which can be Fe, Mn or Cu and Zn (del Río *et al*, 2003; Romero-Puertas *et al*, 2007), thereby producing O_2 and H_2O_2.

Quite some research has already been conducted on the impact of O_3 on antioxidant levels or enzymatic activities of related enzymes but in most cases plants were exposed for a short time to relatively high doses of O_3. Our project, on the contrary, focussed on a more realistic chronic O_3 exposure to moderately elevated concentrations during the entire crop growth. Therefore fresh leaf samples were taken at two occasions during crop growth, one in the vegetative (S1) and one during the generative growth stage (S2), shortly after flowering for oilseed rape and at harvest for broccoli. For each species and each growing season, sampling dates are summarised in Table . A three way ANOVA was performed to analyze metabolite results with growing season, O_3 treatment and sampling time as factors. The significance of the O_3 effect was further evaluated with a two way ANOVA for each individual sampling time, with year and O_3 as factors. For statistical analysis of enzyme activity and gene expression a two sample t test was performed to compare values of O_3 exposed leaves with the NF treatment. As a reference, to compare the O_3 effects at different levels and sampling points, the average antioxidant levels and enzyme activities of leaves sampled at the vegetative stage S1 in the unfiltered OTC (NF) was used (Table XI).

Table X: Overview of sowing date, occurrence of 50% emergence (oilseed rape), planting date (broccoli) and start of O_3 treatment. S1 and S2 are the sampling dates for biochemical analysis during vegetative (S1) and generative (S2) growth.

	Brassica napus					*Brassica oleracea*				
year	Sowing	50%	O_3	S1	S2	Sowing	planting	O_3	S1	S2
2007	18/04	09/05	25/05	04/06	27/06	02/05	13/06	13/06	05/07	30/07
2008	10/04	21/04	07/05	27/05	25/06	07/05	04/06	09/06	10/07	07/08
2009	01/04	11/04	15/04	28/05	25/06	05/05	08/06	12/06	13/07	10/08

Table XI: Average values for measurements of antioxidants and enzyme activities in leaves of oilseed rape and broccoli in NF treatment at S1 for broccoli and oilseed rape

	Brassica oleracea	*Brassica napus*	
TOC	25.1	49.4	µg g FW^{-1}
rASC	2.6	1.6	µmol g FW^{-1}
tASC	3.6	2.5	µmol g FW^{-1}
rGSH	0.11	0.11	µmol g FW^{-1}
tGSH	0.33	0.36	µmol g FW^{-1}
APX	14.0	4.5	µmol ASC mg $protein^{-1}$ min^{-1}
DHAR	21.9	13.7	µmol GSH mg $protein^{-1}$ min^{-1}
MDHAR	5.1	5.3	µmol NADH mg $protein^{-1}$ min^{-1}
GR	5.2	3.9	µmol NADPH mg $protein^{-1}$ min^{-1}
SOD	194.1	205.4	U mg $protein^{-1}$
POX	64.2	147.0	µmol pyrogallol mg $protein^{-1}$ min^{-1}
TAC	3.38	2.28	µmol Trolox g^{-1} FW

TOC: tocopherol; rASC: reduced ascorbate; tASC: total ascorbate (oxidised + reduced); rGSH: reduced glutathione; tGSH: total glutathione (oxidised + reduced); APX: ascorbate peroxidase; DHAR: dehydroascorbate reductase; MDHAR: monodehydroascorbate reductase; GR: glutathione reductase; SOD: superoxide dismutase; POX: unspecific peroxidase; TAC: total (water soluble) antioxidative capacity

Oilseed rape: antioxidants, enzyme activity and gene expression

During the vegetative stage no significant changes in antioxidants levels were found (Figure 22), but after flowering the levels of rASC and tASC as well as rGSH were significantly increased by the O_3 treatment. The highest antioxidant concentrations were found in the NF++ treatment.

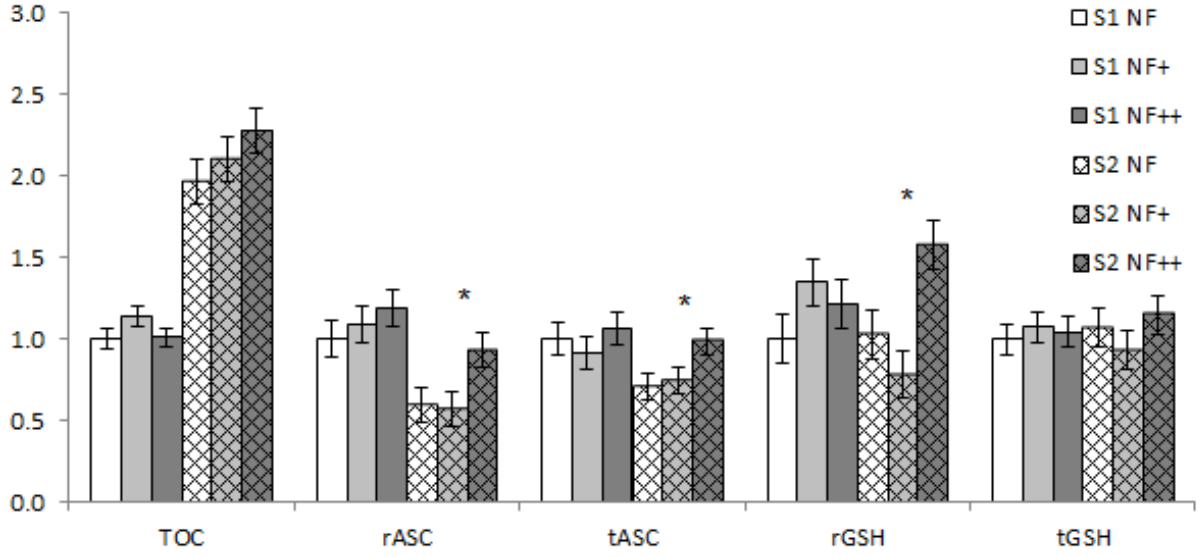


Figure 22: Relative antioxidant levels for TOC, rASC, tASC, rGSH, tGSH in leaves of oilseed rape. Values are averaged for 2007, 2008 and 2009, bars indicate the standard errors (n=9). The relative values were obtained by dividing the actual value by the corresponding antioxidant level in the NF treatment at S1 (*: $p < 0.05$)

At S1 a significant increase in enzymatic activity was seen for SOD in both treatments. This effect was confirmed after flowering (S2), though not significantly (Figure 23). APX and MDHAR showed a significant increase in activity at S2 in both O_3 treatments. For POX a tendency to increase was found at S1 ($p = 0.10$) and S2 ($p = 0.08$) (Figure 23). For GR and DHAR no changes whatsoever were observed.

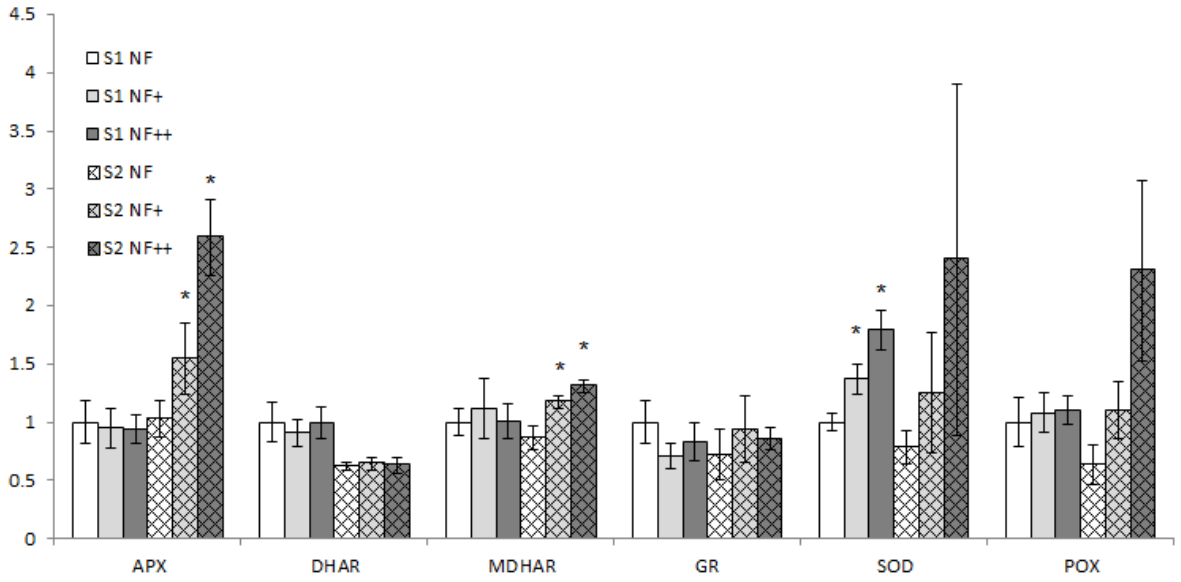


Figure 23: Relative enzymatic activity for APX, DHAR, MDHAR, GR, SOD and POX in 2008 with their standard errors (n=3). All values are devided by the corresponding mean of the activity in the NF treatment at S1 (* $p < 0.05$). The relative values were obtained by dividing the actual value by the corresponding activity level in the NF treatment at S1 (*: $p < 0.05$)

Averaged over all three growing seasons, no significant differences in total water soluble antioxidative capacity (TAC) were detected between the different O_3 treatments (Figure 24).

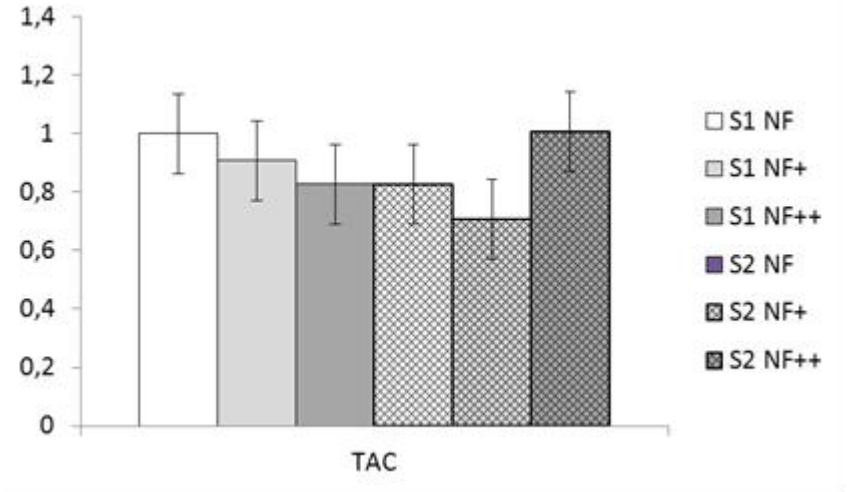


Figure 24: Relative TAC (total water soluble) antioxidative capacity in leaves of oilseed rape. Values are averaged for 2008 and 2009, bars indicate the standard errors. The relative values were obtained by dividing the actual value by the corresponding antioxidant level in the NF treatment at S1 (ns $p > 0.05$)

The fold change from different coding sequences for APX (Figure 25a) varied considerably between sequences and treatments. Only in the NF++ treatment a significant decrease in expression was found in two of six tested genes, one in the early growing stage and one in the later stage. In general a down regulation of APX coding sequences was observed. For GR (Figure 25 b) and SOD (Table XII), two and one coding sequence was evaluated respectively. There were no significant differences in gene expression levels. For MDHAR (Figure 25 c) a significant up-regulation was seen at the later stage in the NF++ treatment in one of two tested sequences. For DHAR (Figure 25 d), there was a significant decrease in gene expression at the latest sampling time in both treatments for one out of three tested sequences. For SOD and for ASC, GSH and TOC synthesis, gene expression fold changes for the last sampling point from different enzymes in the pathway are summarized in table XII. No significant changes were found. For the first sampling point one γ-TMT and one phytolkinase, both involved in TOC-synthesis, were respectively down- (p=0.07) and upregulated (p=0.08), but only slightly.

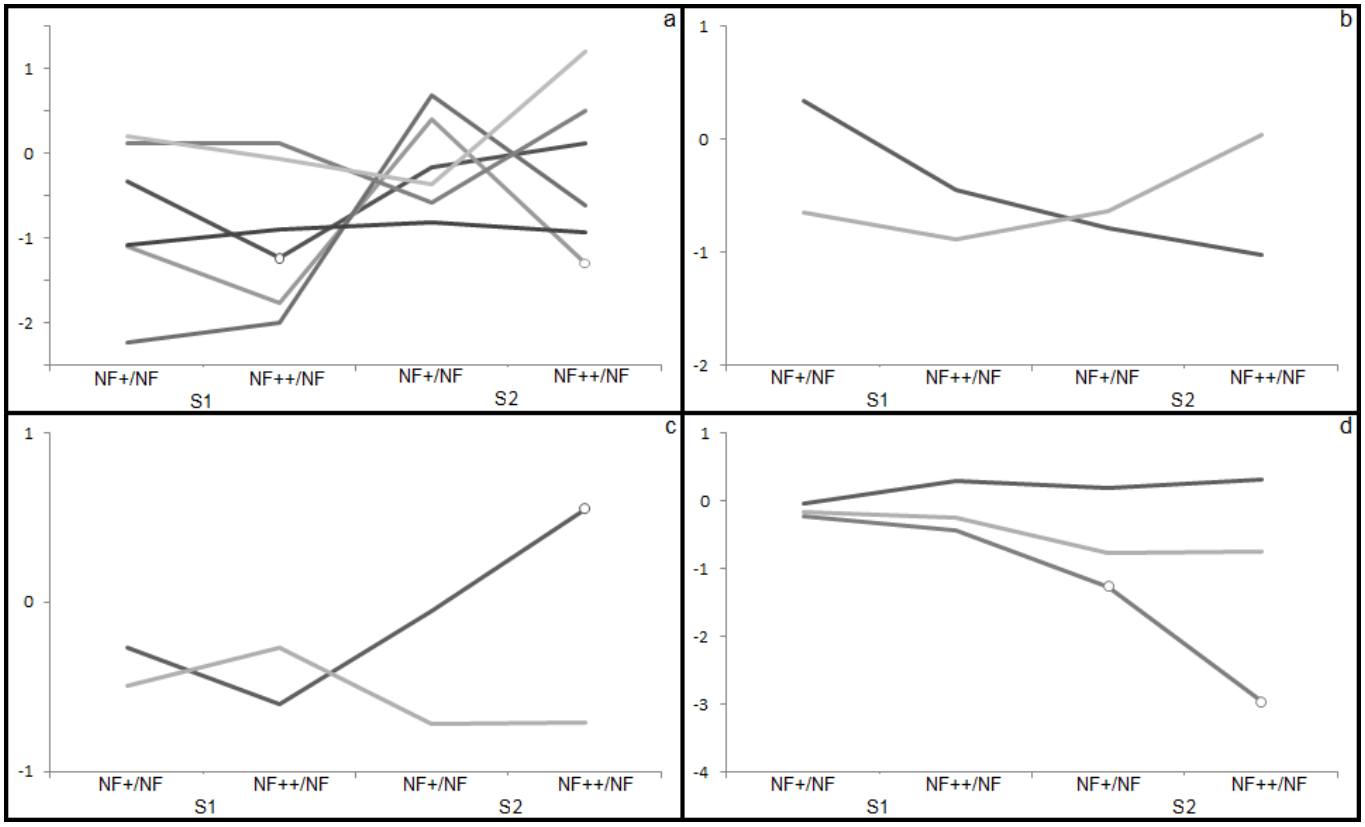


Figure 25: Log_2(Fold change) after RT-PCR in the Y-axis are represented for different isoforms of APX (a), GR (b), MDHAR (c) and DHAR (d) in oilseed rape. Open symbols represent significant changes in gene expression ($p < 0.05$)

Table XII: Log_2(Fold change) at S2 of genes involved in ASC, GSH and TOC biosynthesis and SOD for oilseed rape (both treatments) and broccoli (NF++). Significantly altered fold changes are underlined. *At4g26850 encodes for a protein with L-galactose guanyl transferase activity **T16L1.160 encodes a L-galactose dehydrogenase

	B napus		*B oleracea*
	NF+	NF++	NF++
Superoxide dismutase	-1.02	-0.92	-0.27
GDP-mannose pyrophosphorylase 1	-0.59	-0.58	-0.48
GDP-mannose pyrophosphorylase 2	-0.88	-0.89	-0.68
GDP-mannose 3,5-epimerase 1	-0.74	-0.30	-0.60
GDP-mannose 3,5-epimerase 2	-0.93	-0.58	-1.53
Putative uncharacterized protein At4g26850	-1.26	-1.06	-1.31
Putative uncharacterized protein T16L1.160	-0.06	-0.03	-1.30
L-galactono-1,4-lactone dehydrogenase precursor 1	-0.80	-0.34	-0.77
L-galactono-1,4-lactone dehydrogenase precursor 2	0.35	0.16	-0.52
Glutathione synthetase, chloroplast precursor	-0.15	0.12	-0.54
Tocopherol cyclase, chloroplast precursor	-0.37	0.40	-0.59
Phytol kinase 1, chloroplast precursor	0.46	0.31	0.23
Gamma-tocopherol methyltransferase	-0.32	-0.68	-0.18

Broccoli: antioxidants, enzyme activity and gene expression

At S1 no O_3 induced changes in antioxidants levels of TOC, rASC, tASC, rGSH, tGSH were found (Figure 26), but at S2 the levels of TOC were significantly reduced by elevated O_3. Moreover, in the generative phase the TOC levels were much higher compared to the vegetative stage (Figure 26).

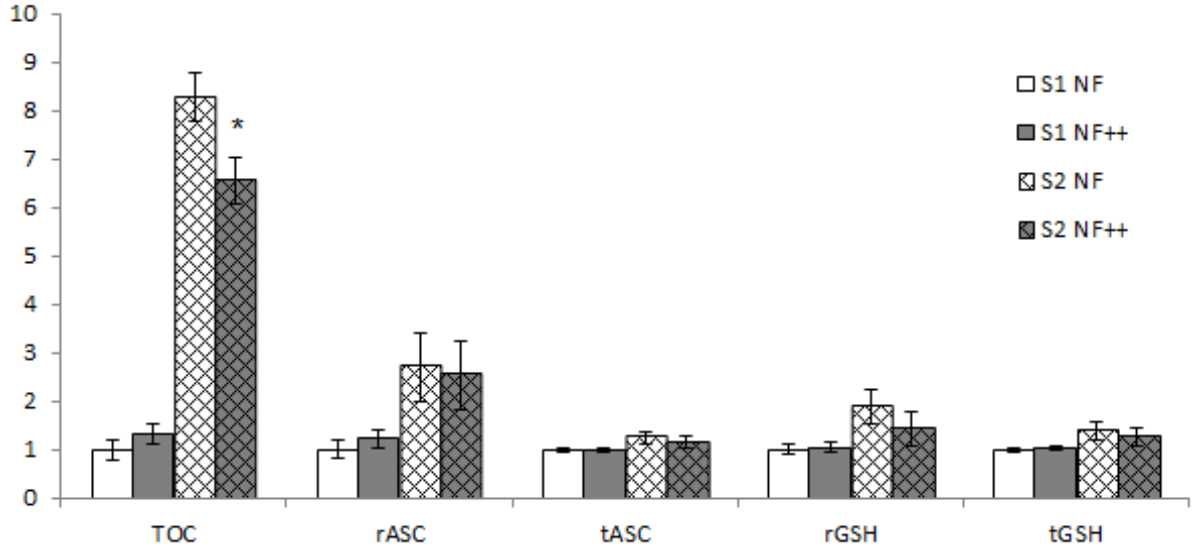


Figure 26: relative antioxidant levels for TOC, rASC, tASC, rGSH, tGSH in broccoli. Values are averaged for 2007, 2008 and 2009 with their standard errors (n=9). All relative values were obtained by deviding the actual value by the corresponding mean antioxidant level in the NF treatment at S1 (*$p < 0.05$)

There were no significant differences in enzymatic activity between NF and O_3 exposed leaves at S1 (Figure 27). For both peroxidases (APX and POX) a significant increase in enzymatic activity was found at S2. At this time no significant changes in enzymatic activity were found for SOD, MDHAR, GR and DHAR, although SOD showed a tendency to decrease at S1 ($p = 0.06$) and S2 ($p = 0.09$) (Figure 27).

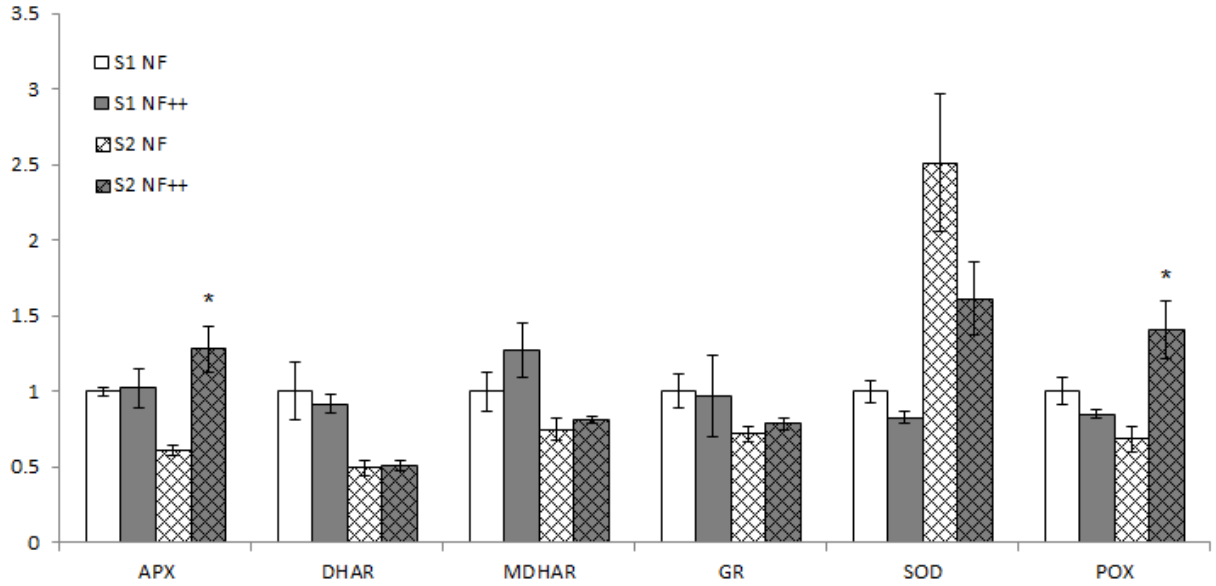


Figure 27: relative enzymatic activity for APX, DHAR, MDHAR, GR, SOD and POX in 2008 with their standard errors (n=3). All values are devided by the corresponding mean of the activity in the NF treatment at S1 (*$p < 0.05$)

Averaged over all three growing seasons, no significant differences in TAC were detected between the different O_3 treatments (Figure 28).

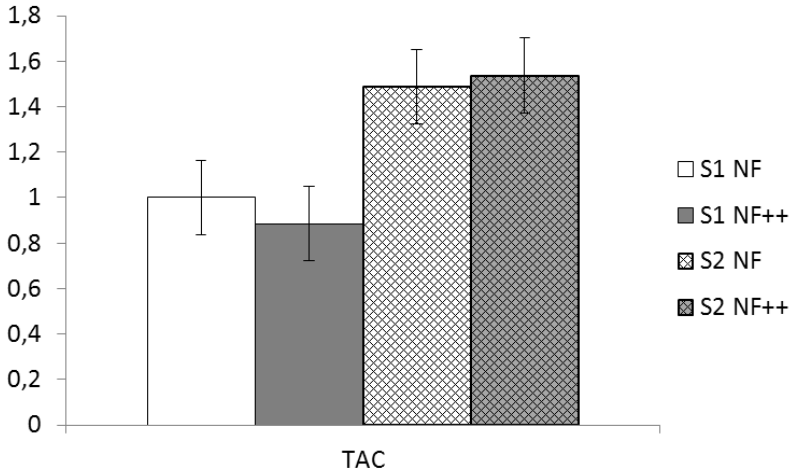


Figure 28: Relative TAC (total water soluble) antioxidative capacity in broccoli leaves. Values are averaged for 2008 and 2009, bars indicate the standard errors. The relative values were obtained by dividing the actual value by the corresponding antioxidant level in the NF treatment at S1 (ns $p > 0.05$)

Figure 29 shows gene expression levels for APX, GR, MDHAR and DHAR. For APX (Figure 29 a) upregulation was seen at S1 for six tested sequences, with two significantly upregulated genes. Upregulation is considerably lower at S2, or shifted towards a downregulation. In one sequence, O_3 induced a significant downregulation at S2. For GR (Figure 29 b) no significant changes were seen and for one of two MDHAR sequences a significant downregulation was found at S2. For DHAR an upregulation of both tested sequences was found at S1, but this phenomenon disappeared at S2. In general, gene expression ratios are smaller in S2 compared to S1. Regulation of SOD was not significantly altered by O_3 exposure (Table XII). For ASC biosynthesis (Table XII), two genes were found to be downregulated by O_3 exposure at S2: GDP-mannose pyrophosphorylase and L-galactose dehydrogenase. For TOC-biosynthesis (Table X) another gene was significantly downregulated at S2, namely TOC cyclase, this is complemented with a significant downregulation of TOC cyclase and phytolkinase at S1 (data not shown). All biosynthesis genes were downregulated at S2 in broccoli.

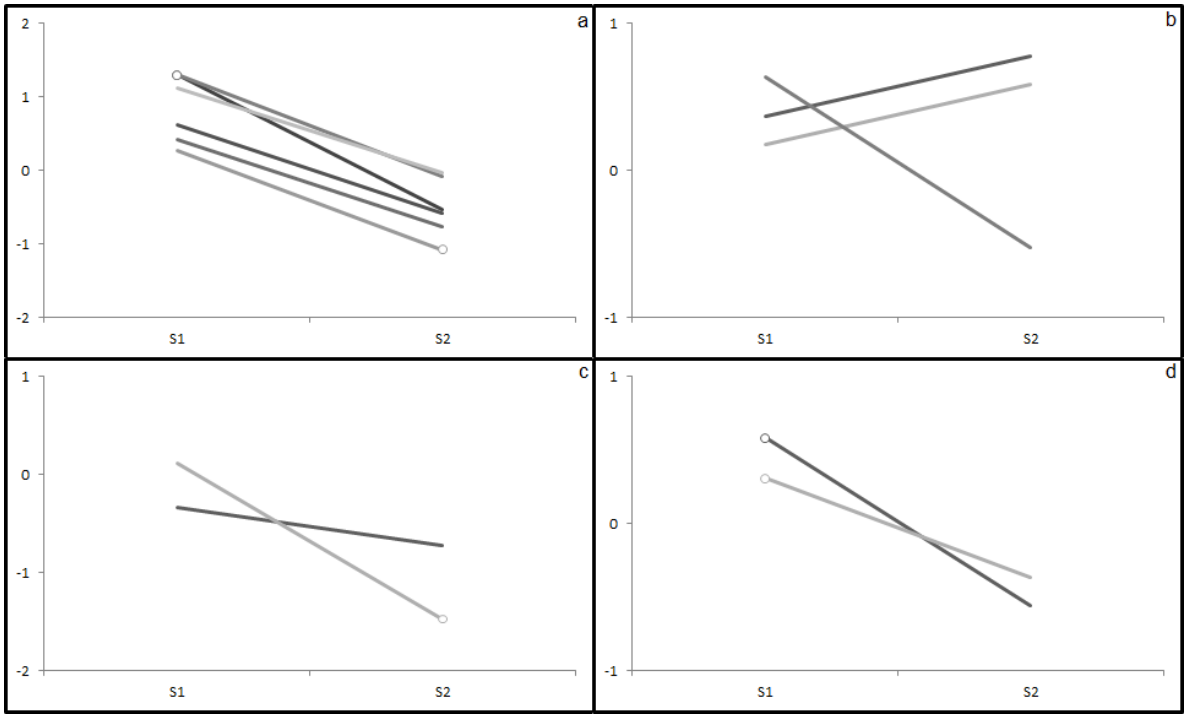


Figure 29: Log_2(Fold change) from RT-PCR (NF++/NF) in the Y-axis are represented for different genes for APX (a), GR (b), MDHAR (c) and DHAR (d) for broccoli. Open symbols represent significant changes in gene expression ($p < 0.05$)

Conclusions

Long term exposure to moderately elevated O_3 induced biochemical changes in *Brassica* species, but the two investigated species, broccoli and oilseed rape, clearly showed different responses with regard to the antioxidative defence mechanisms. In general, there was no significant change in the antioxidant levels in the leaves during the vegetative growth stage. However, at the generative stage, after a longer period of O_3 exposure, antioxidant levels were indeed altered. In oilseed rape, O_3 induced an increase in water soluble ASC, total and reduced, and GSH. This phenomenon was not observed in broccoli, but there was a decrease in α--TOC. It is suggested that O_3 or ROS may have more difficulties to penetrate the lipid fraction of broccoli cells, where it can be partially disintegrated by oxidation of TOCs and lipid peroxidation is avoided by oxidation of TOCs (Munné-Bosch, 2005; Maeda and DellaPenna, 2007). Also

the downregulation of TOC cyclase in broccoli can be responsible for the decrease in α-TOC concentrations. In the leaves of both oilseed rape and broccoli, TOC concentrations increase over time. In broccoli, no other antioxidants concentrations changed compared to oilseed rape, where O_3 induced an increase in the production of water soluble antioxidants in the cell. This increase in ASC and GSH at S2 in oilseed rape is reflected in the TAC at this point, also the increase of ASC over time in broccoli is reflected in the TAC measurement. Thus confirming the importance of ASC as most abundant pool of metabolites with redox capacity (Noctor and Foyer, 1998)

The more sensitive oilseed rape (De Bock *et al.*, 2011) showed a decrease in rASC (reduced ASC) over time, whereas in broccoli ASC increased over time. This confirms the importance of reduced ASC in O_3 tolerance (Robinson and Britz, 2000). However, in broccoli both DHAR and MDHAR activity did not change over time, but the redox status was increased. In oilseed rape, DHAR activity decreased over time, thus explaining the decrease in rASC, but DHAR activity was not influenced by O_3. Consequently the higher rASC concentrations can be explained by the increased MDHAR activity. This correlates well with the upregulation of at least one isoform of MDHAR. In addition; the concentration of rASC can be influenced by an altered balance between biosynthesis of ASC and breakdown of DHAR. In oilseed rape, no significant changes in gene expression levels of genes involved in the biosynthesis pathways of both ASC en GSH were found in response to O_3 exposure. If any regulation, these genes show some downregulation, but concentrations of both metabolites increased. In broccoli, some genes involved in ASC biosynthesis were downregulated, however, concentrations of both GSH and ASC were stable. Both in oilseed rape and broccoli, the O_3 exposure clearly activated the enzymes that are in direct contact with ROS species. This activation of primary antioxidative enzymes is seen both over time and as a consequence of the O_3 treatment. This gives a clear indication that O_3 treatment causes an important oxidative stress on these plants. An increase in POX and APX is seen in both species and also SOD increases in oilseed rape, but in broccoli this did not occur as a consequence of the O_3 treatment. Also for many other parameters, broccoli seems less affected by the O_3 treatments in comparison to oilseed rape (De Bock *et al*., 2011). POX and APX can rapidly detoxify ROS species but no up regulation of these enzymes was found at the transcriptomic level for both species in samples taken at the generative growth stage where APX activity clearly increased. Therefore activation of these enzymes is probably regulated post-transcriptionally (Madhusudhan *et al*, 2003). It is remarkable that the higher APX activity did not result in a lower reduced ASC or GSH, taken into account that DHAR and MDHAR activity was unaltered in broccoli. Activity of both enzymes was probably high enough to keep the reduced antioxidant pool at a suitable level. In oilseed rape MDHAR activity was slightly increased resulting in higher rASC in the NF++ treatment.

Whether regulation of enzyme activity is regulated transcriptomically or posttranscriptomically is not always clear. For GR no regulation was found at the transcriptomic levels and no changes in activity were detected. DHAR activity did not change in either of the plant species, however in broccoli at the vegetative stage an upregulation was seen whereas a downregulation of the different DHAR genes was observed in oilseed rape at the generative sampling point. Downregulation of one isoform of MDHAR in broccoli did not influence MDHAR activity. It is however the other isoform that was upregulated in oilseed rape. For SOD and other genes listed in table XII, conclusions must be handled with care as only few isoforms out of many were successfully measured with RT-PCR.

3.6.2 Chronic O_3 effects on leaf GSL concentrations

Oilseed rape

Oilseed rape leaves mainly contained indolic GSLs, but for both fractions the concentrations decreased as the growing season progressed and leaves became more senescent (Figure 30). This also influenced the ratio of aliphatic/indolic GSLs. In comparison to the seeds, the concentrations in the leaves were about five to six times lower and the relative proportion of indolic GSLs was lower (cfr Table VII). After flowering, the fraction of indolic GSLs that remained in the leaves was significantly higher in the O_3 exposed plants. Also large year to year variations were observed, but no year x O_3 interactions.

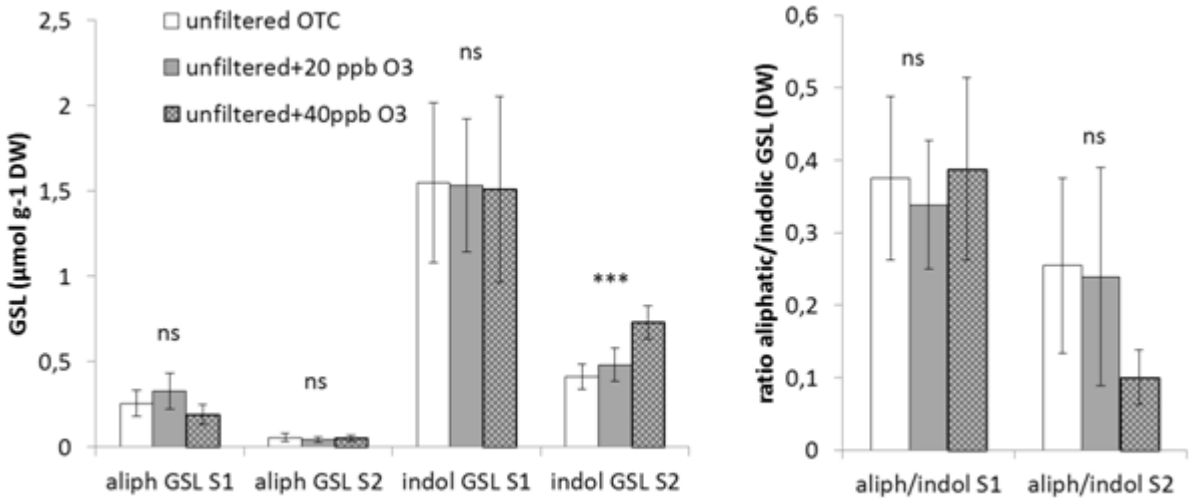


Figure 30: Mean concentrations of aliphatic and indolic GSLs (2007-2008-2009) in upper canopy leaves of oilseed rape and the ratio of the aliphatic/indolic fraction, sampled in July at the end of the vegetative stage (S1) and in August after flowering (S2). *** $p < 0.001$; ns $p>0.05$ '(two-way ANOVA)

Broccoli

In broccoli vegetables, O_3 induced a significant increase of the ratio aliphatic/indolic GSL due to an increase in aliphatic GSLs glucoiberin and glucoraphanin accompagnied by a decrease in the indol GSLs glucobrassicin and neoglucobrassicin (Figure 15 & 16). In broccoli leaves, sampled at the appearance of the florets (July) and at harvest (August), the same tendency is observed, though not significant (Figure 31). In the leaves the ratio aliphatic/indolic GSLs increases with leaf age, but remains well under the ratio in the vegetables (**Error! Reference source not found.** cfr figure 16). The tendency to decreased indolic GSL concentrations in leaves as a response to elevated O_3 may be a consequence of enhanced senescence, as this decrease is also observed in older leaves (Figure 31).

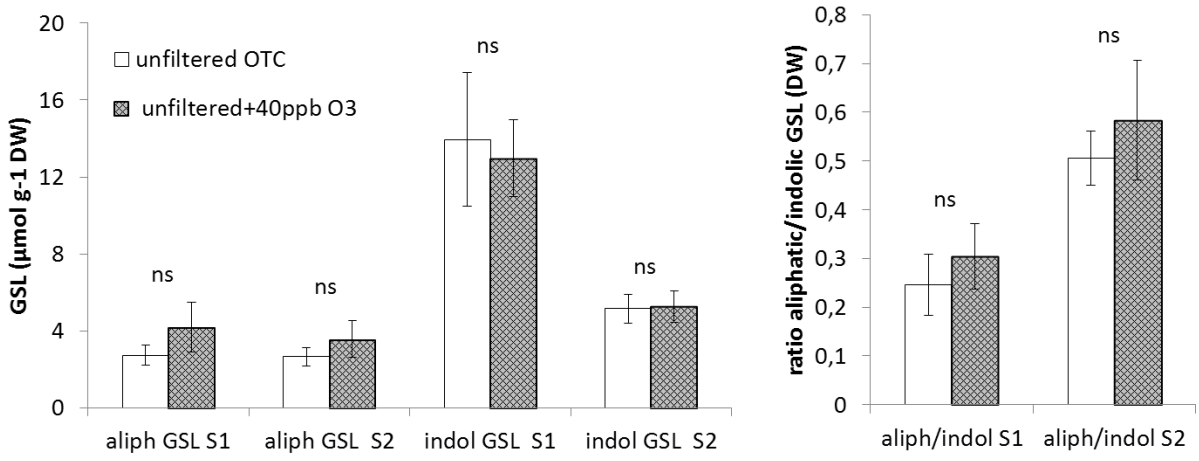


Figure 31: Mean concentrations of aliphatic and indolic GSLs (2007-2008-2009) in upper canopy broccoli leaves and the ratio of aliphatic/indol GSLs sampled before the appearance of the florets in July (S1) and at harvest in August (S2). Differences between NF and NF++ were not significant (two way ANOVA $p > 0.05$)

3.6.3 Overview of chronic O_3 effects on gene expression levels (microarray)

At S1 (vegetative growth stage) a cross species two-way ANOVA resulted in 661 genes that were significantly ($p < 0.01$) influenced by the O_3 treatment. This gene set was divided into five clusters after normalization of the intensity values and for each cluster overrepresentation of genes was investigated using cytoscape (2.6) with the BINGO plugin (2.4). Only for the fifth cluster, containing 83 genes (of which 78 recognized in BINGO) which are downregulated in both oilseed rape and broccoli, significant overrepresentation of genes (against the group of 18489 genes of which 17852 are recognized, because some *Arabidopsis* genes are not in the gene list of BINGO) was found. Two genes (AT1G20693 and AT1G20696) out of five total genes of the structural constituent of chromatin, were found to be downregulated in *Brassica* species. Also two genes (AT2G29400 and AT1G64040) out of five of the protein phosphatase type 1 complex were found. Each species was thereafter analyzed separately. For broccoli, significant differences for each gene at S1 were determined with a two sample T-test. A total of 278 genes were retained ($p < 0.05$), of which 275 (261 recognized) were downregulated and only three were upregulated. The downregulated genes were tested for overrepresented gene ontologies: 14 (AT1G02340, AT4G37870, AT1G43700, AT3G17810, AT4G00730, AT5G12200, AT1G31930, AT1G56590, AT3G01090, AT2G21410, AT5G45710, AT2G25930, AT2G47000 and AT5G01220) out of 171 genes related to response to external stimulus where found. This means that O_3 did indeed trigger a defence response in broccoli plants. For oilseed rape, significant regulation at S1 was determined by ANOVA ($p < 0.05$). A total of 512 genes (489 recognized) were subsequently divided into six clusters. In the sixth cluster with 39 upregulated genes (38 recognized), nine out of a total of 95 photosynthesis-related genes are found (AT4G09650 AT2G06520 AT4G03280 *AT4G12800* *AT1G30380* AT5G66190 AT3G54890 *AT1G55670* and *AT1G08380*) of which four out of 13 are related to photosystem I (indicated in *italic*). Also in other clusters, two more photosynthesis genes of which one related to photosystem I (*AT4G02770* and AT5G47110) were upregulated, but only in NF++. Also two photosynthesis genes were significantly downregulated (AT4G04040 and AT1G50250)

At S2 (generative growth phase) a cross species two-way ANOVA yielded 588 significantly regulated genes ($p < 0.05$), however no clusters were found with an overrepresentation of gene ontology

categories. A one-way ANOVA of oilseed rape gene expression at S2 yielded 544 significantly regulated genes ($p < 0.05$), but it was impossible to divide those genes into clusters with overrepresentation of certain gene categories. Also when both gene regulations at S1 and S2 were combined for oilseed rape in a two-way ANOVA, resulting in 488 genes ($p < 0.05$), it was impossible to divide into clusters with significant overrepresentation of gene ontology categories. For broccoli, gene expression at S2 was examined with a two sample T test, both clusters of the resulting 173 genes ($p < 0.05$) showed no overrepresentation of certain gene categories. Finally, also for broccoli S1 and S2 were combined into a two-way ANOVA, resulting in 1890 genes ($p < 0.05$) or 814 at ($p < 0.01$), but no significant overrepresentation was established.

As a microarray consists of several thousands of tests performed at once, significance levels of $p < 0.05$ and $p < 0.01$ are insufficient to determine differences in gene expression levels. These p-values are too low to rule out the possibility of falsely determined significant regulations, for some of the many genes. A multiple testing correction should be applied. However, for these microarray experiments, this would lead to none or only a few significantly regulated genes, which would not have been enough to perform a good cluster analysis. Even without multiple testing, only a relatively small number of genes was significantly up- or downregulated in these *Brassica* species, which is possibly caused by the low stress levels induced by the chronic O_3 exposure after just a few weeks, as also no physiological or biochemical changes are seen at this point in time. Interestingly a relatively large set of nine genes of *Brassica napus,* linked to photosynthesis, was significantly upregulated in both treatments, and consequently were categorized in the same cluster, containing only 39 genes (Figure 32). Most of these genes are linked to different subunits of PS I. The overrepresentation of these photosynthesis genes is clearly visualized for both treatments with MapMan (Figure 33, Figure 34) where the LOG_2 (Fold Change) of all significantly regulated genes are spotted against all metabolic-related genes. However, it is emphasized that no changes in photosynthesis or in yield was seen at this point in time, it is only later that photosynthesis was decreased, and not upregulated, in NF++ and NF+ treatment. No other pathways with a similar overrepresentation were visible on the metabolic representation (Figure 33, Figure 34). A closer look on the light reactions pathways again showed the upregulation of PS I genes in both treatments (Figure 35, Figure 36**Error! Reference source not found.**). Based on the metabolic pathway it can then be hypothesized that a boost in photosystem I genes can lead to an enhanced production of NADPH (Peltier *et al*, 2010). This is an important energy source that is known to be utilized in different stress responses to control or minimize damage to the cell. More specifically, O_3 can be reduced by ASC, with APX and oxidized ASC can be reduced by oxidation of GSH, which can in its turn be reduced by NADPH (Halliwell and Asada, cycle, Noctor *et al*, 1998. Therefore, it is possible that a boost in NADPH production can lead to a boost in O_3 degradation capacity, by natural regeneration of ASC. Indeed, measurements of ASC at the same point in time did not show a decrease in rASC. If O_3 toxicity is effectively countered by an increase in NADPH, it is then also understandable, that only regulation of relatively few other genes is significantly affected. This is also explained by the time of sampling of samples for the microarray experiment, which was in the vegetative stage when there was no visible or physiologically measurable O_3 damage as a consequence of the chronic O_3 exposure. The results of analysis of only *Brassica oleracea* or the cross species analysis did not show any remarkable results.

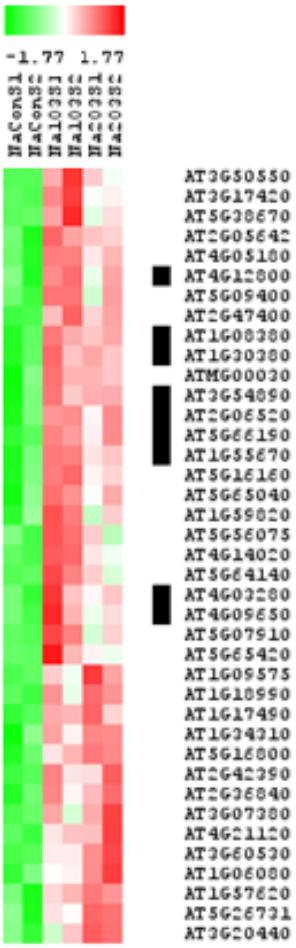


Figure 32: Heath map of cluster 6 with 39 significantly regulated genes and their normalized intensity values in the six oilseed rape samples from green (low intensity) to red (high intensity), photosynthesis genes are marked (black squares)

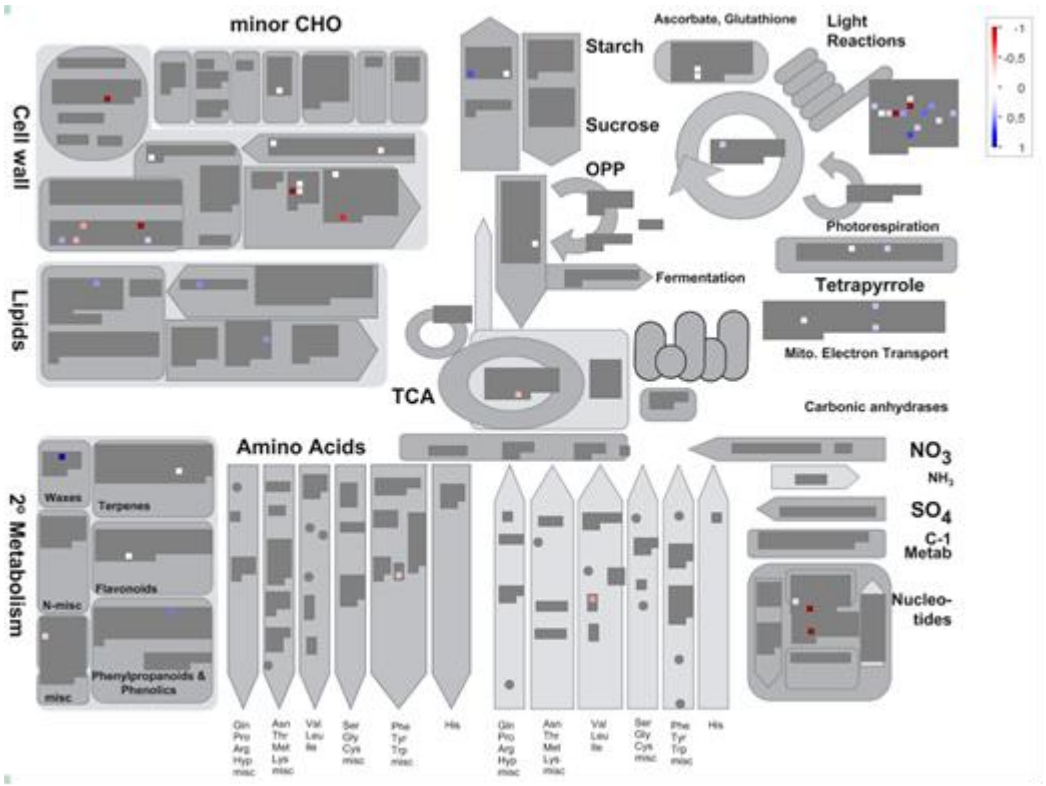


Figure 33: Overview of changes in regulation of metabolism of *Brassica napus* for NF+ treatment compared to NF treatment. LOG_2(Fold Change) values of significantly regulated genes are color coded from blue (down) to red (up), not significantly regulated genes are in gray.

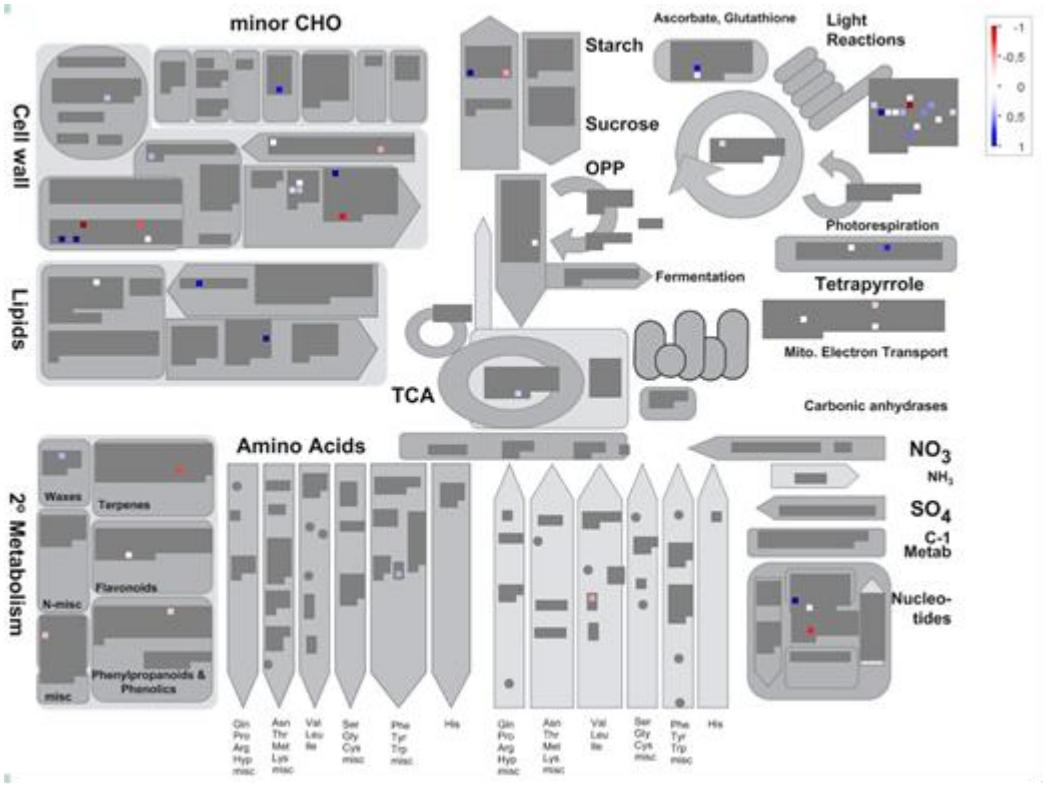


Figure 34: Overview of changes in regulation of metabolism of *Brassica napus* for NF++ treatment compared to NF treatment. LOG_2(Fold Change) values of significantly regulated genes are color coded from blue (down) to red (up), not significantly regulated genes are in gray.

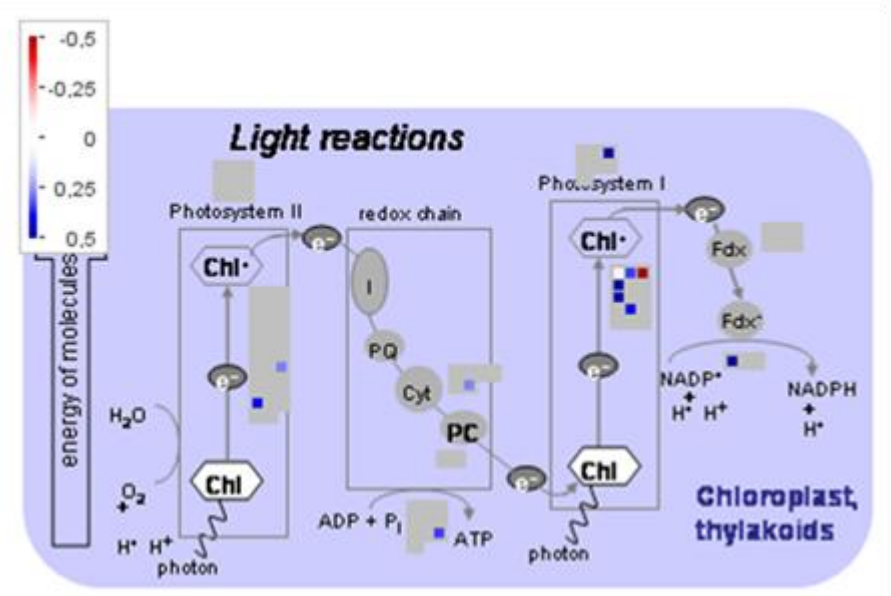


Figure 36 Overview of regulation of genes of *Brassica napus* linked to photosynthesis in NF+ treatment compared to NF treatment. LOG_2(Fold Change) values of significantly regulated genes are color coded from blue (down) to red (up), not significantly regulated genes are in gray

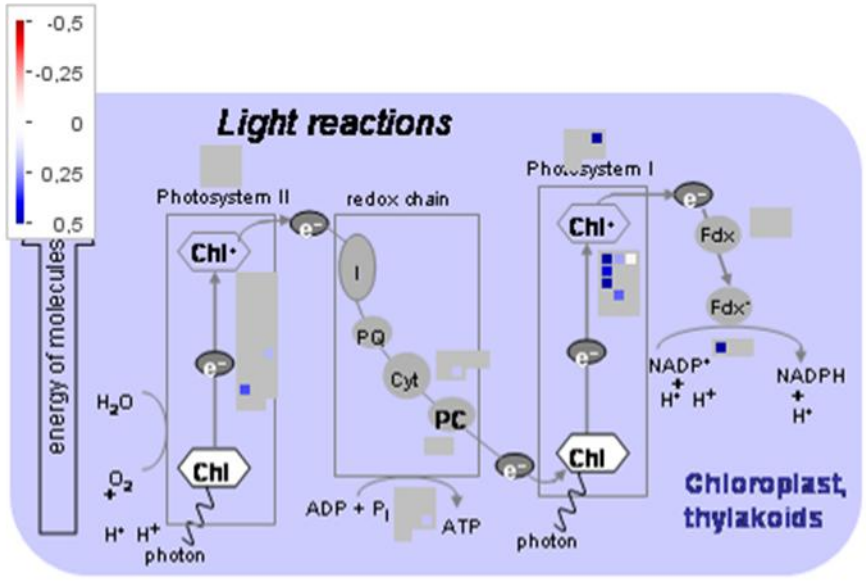


Figure 37: Overview of regulation of genes of *Brassica napus* linked to photosynthesis in NF++ treatment compared to NF treatment. LOG_2(Fold Change) values of significantly regulated genes are color coded from blue (down) to red (up), not significantly regulated genes are in gray.

The data of the microarray experiment, with a special interest in the PS I genes were, however, not confirmed by RT-PCR analysis. Therefore, and because of the lack of significance in the microarray results, all of the above results should be considered with care. The main reason for the lack of confirmation with RT-PCR can probably be attributed to the low significance levels of the microarray results. Both RT-PCR and microarray analysis are limited in the possibility of doing many repetitions. In addition we are dealing with samples taken from field experiments, where variability between plants is higher. This automatically results in a small number of significant results, whereas significant results of the microarray are not necessary significant when measured in a RT-PCR set-up, because analysis was done on different samples. This should not be a problem in *in vitro* experiments, but proved to be a problem in these field experiments.

3.6.4 Specific results of chronic O_3 effects on gene expression levels (quantitative PCR)

In total around 80 genes of which three housekeeping genes were tested with RT-PCR on different samples harvested in 2008 and 2009 for broccoli and oilseed rape. Results of antioxidant-related genes were already presented in the previous chapter. Table XIII gives an overview of all other genes that were found to be significantly up- or downregulated. It is striking that the list is very short, compared to the large number of genes that were tested.

Table XIII: Overview of all significantly altered gene expressions determined with RT-PCR (* $p < 0.05$, ** $p < 0.01$)

	involved in	ratio	SE	Sign
oilseed rape				
Vegetative				
peroxidase 43 precursor	Peroxidase	1.64	0.38	*
Generative				
Myrosinase (1)	GSL degradation	2.11	0.30	**
Cytochrome P450 79B3	indol GSL synthesis	2.03	0.62	*
Myrosinase binding protein	GSL degradation	1.70	0.30	*
Broccoli				
Vegetative				
Myrosinase (1)	GSL degradation	2.02	0.48	*
Cytochrome P450 79B3	indol GSL synthesis	3.28	0.59	*
Generative				
Myrosinase (2)	GSL degradation	0.39	0.06	**

3.6.5 Conclusions on O_3 induced changes in leaf metabolism

Chronic O_3 exposure has an influence on leaf antioxidant levels of both *Brassica* species at the end of the growing season, however, oilseed rape and broccoli respond differently. In general, in oilseed rape there was an increase of the antioxidant level: total and reduced ASC, as well as rGSH concentrations were increased and the same tendency was observed for tGSH and α-TOC (not significant). To

maintain a higher amount of rASC in O_3 treated oilseed rape plants, MDHAR activity is increased through the upregulation of the corresponding gene. The TAC follows the observations that were made in ASC measurements for both species. In broccoli no changes in leaf oxidants were observed in response to O_3, neither before bud formation nor at harvest, with the exception of α-TOC that was decreased as a result of the season long elevated O_3 exposure (but increased with plant/leaf age). A downregulation of some genes important for TOC biosynthesis in broccoli could contribute to the lower levels of TOC measured at the end of the growing season (Figure 33). In accordance with the observed shift in GSL composition of the broccoli vegetables, the ratio aliphatic/indolic GSLs in the leaves also showed a tendency (not significant) to increase under elevated O_3.

Antioxidative defence mechanisms of broccoli and oilseed rape in response to chronic O_3 exposure are characterised by an increase of the activities of enzymes that are directly involved in the detoxification of ROS species, whereas the activities of enzymes involved in antioxidant regeneration generally remain unaltered. Regulation of APX activity is done posttranscriptionally. In oilseed rape the upregulation of genes related to PS I was already observed after just a few weeks of O_3 exposure, however this could not be confirmed by RT-PCR analysis. An increase in NADPH levels would be usefull in these plants for the prevention and repair of O_3 damage within the cell, e.g. by reduction of antioxidants, such as ASC. The maintenance of the redox state of ASC levels in broccoli leaves (Figure 34) could be caused by the upregulation of DHAR, but this was not reflected in increased activities of these enzymes.

4 POLICY SUPPORT

Ozone has long been recognised as causing losses in crop productivity and changes in the quality of agricultural products. There is now a strong demand from policy makers for the quantification of O_3 damages to be fed into cost-benefit analysis of emission control strategies (Holland *et al*, 2006). This project supplied such information for some major *Brassica* crops: *Brassica napus* (oilseed rape) and *Brassica oleracea* (broccoli).

Economic losses are expected for oil seed rape if O_3 concentrations continue to rise. In comparison to the current situation, seed yield losses of spring oilseed rape may be reduced by 30% within 100 years if future ambient 7 or 12 hr average O_3 concentrations increase to a range of 51 – 75 ppb, as predicted by Assessment Report Four (IPCC, Meehl *et al*, 2007). Oil yield is even more affected due to an additional decrease of the oil percentage which will need to be taken into account for the estimation of biofuel production under future scenarios of increasing tropospheric O_3 levels. Based on our data the critical AOT40 (Accumulated Ozone exposure over a threshold of 40 ppb) to prevent 5% seed or oil yield are respectively 3.7 and 3.2 ppm h from emergence until harvest, which implies that the presently accepted critical level of 3 ppm h for agricultural crops (UNECE) will also protect spring oilseed rape.

For the quantification of O_3 responses the flux-based method is preferred on the grounds that it estimates yield losses and quality effects against received dose of O_3, rather than against simple exposure to ambient levels. However, so far the flux-based method could only be applied to wheat and potato. We now developed such a model for spring oilseed rape and broccoli so that a wider range of crops may be included in the O_3 risk assessment. The flux-based critical level above which 5% yield reduction for oilseed rape may be expected, is estimated at a POD_6 (Phytotoxic Ozone Dose above a threshold of 6 nmol s^{-1} m^{-2} projected leaf area (PLA)) of 4.4 mmol m^{-2} (for seed production) and 3.9 mmol m^{-2} PLA (for oil production).

These concentration- and flux-based critical levels can be compared to modelled O_3 concentrations and fluxes for 50 km x 50 km grid squares across Europe as supplied by EMEP (European Monitoring and Evaluation Programme) to identify those areas that are most at risk for O_3 damage to oilseed rape, at present but also as predicted for the future.

This project also illustrates that the focus on yield changes could however result in a misleading risk assessment and economic extrapolations since also qualitative attributes of the harvested products may be affected by O_3. Depending on nature of these quality traits for industrial processing and consumer's health, the consequences of increasing tropospheric O_3 concentrations may have beneficial or detrimental consequences on the food and feed chain.

5 DISSEMINATION AND VALORISATION

- Poster presented at the Workshop on Environment & Health. 15&16/01/2007. Brussels, Belgium

"Impact of tropspheric ozone on food and feed quality of *Brassica* species" - Vandermeiren K., Horemans N., Gielen B.

- Poster presented at the NecoV wintersymposium. 7&8/02/2008. Antwerp, Belgium

"Impact of tropospheric ozone on growth of *Brassica napus* and *Brassica oleracea*" - De Bock M., Horemans N., Gielen B., Ceulemans R., De Temmerman L., Vandermeiren K.

- Oral presententation at 7th Workshop on Sulfur Metabolism in Plants. 13-16 May 2008. Warsaw, Poland

"Impact of troposheric ozone on glucosinolate and vitamin C content of oilseed rape and broccoli" – K.Vandermeiren, M. De Bock, N. Horemans, B. Gielen.

- Oral presentation at the APGC Symposium "Plant Functioning in a Changing Global Environment. 7-11 December 2008. Creswick, Australia.

"Impact of tropospheric ozone on yield and quality of *Brassica napus*" – K. Vandermeiren, M. De Bock, N. Horemans, R. Ceulemans, L. De Temmerman.

- Oral presentation at the 22nd Task Force Meeting of the ICP-Vegetation. 2-5 February 2009. Braunschweig, Germany.

"Impact of tropospheric ozone on the food and feed quality of *Brassica* species (OFFQ)" – K. Vandermeiren, M. De Bock, N. Horemans, M. Op de Beeck.
http://icpvegetation.ceh.ac.uk/events/documents/Vandermeirenetal6a.pdf

- Poster presented at the Annual Meeting of the Society-for-Experimental-Biology. 28/6/2009 – 1/7/2009, Glasgow, Shotland

"Impact of tropospheric ozone on food and feed quality of *Brassica* species" - De Bock M, Vandermeiren K, Horemans N, Ceulemans R, Guisez Y

- Press release in Food Navigator, 01-July-2009.

"New research shows ozone's effects on oil seed rape" By Caroline Scott-Thomas. http://www.foodnavigator.com/Science-Nutrition/New-research-shows-ozone-s-effects-on-oil-seed-rape

- Oral presentation at the 15th PhD symposium on applied and biological sciences. 6/11/2009, Leuven, België

"Impact of tropospheric ozone on food and feed quality of *Brassica* species" - De Bock M, Vandermeiren K, Ceulemans R, Guisez Y

- Poster presented at the Expert Panel Meeting. 9-12/11/009. Ispra, Italy.

"A comparison of two stomatal conductance models using data on two *Brassica* species" M. Op de Beeck, M. de Bock, K. Vandermeiren, R. Ceulemans, Y. Guisez, L. De Temmerman

- Oral presentation at the 23rd Task Force Meeting of the ICP-Vegetation. 1-3/02/2010. Tervuren, Belgium

"Which ozone dose response works best for oilseed rape and broccoli?" Op de Beeck M., De Bock M., Vandermeiren K., De Temmerman L., Ceulemans R., Guisez Y.
http://icpvegetation.ceh.ac.uk/events/documents/DeBock_OpdeBeecketal.pdf

- Poster presented at the 24th Task Force Meeting of the ICP-Vegetation. 31/01 – 02/02/2011.

Rapperswil, Switzerland.
"Does ozone exposure lead to biochemical changes in *Brassica* species?" M. De Bock, N. Horemans, Y. Guisez, K. Vandermeiren

6 PUBLICATIONS

6.1 Peer reviewed

- Vandermeiren K., De Bock M., Horemans N., Gielen B. (2009). Impact of tropospheric ozone on glucosinolate and vitamin C content of oilseed rape and broccoli. In: Sulfur metabolism in plants. A. Sirko, L.J. De Kok, S. Haneklaus, M.J. Hawkesford, H. Rennenberg, K. Saito, E. Schnug and I. Stulen (Eds), Backhuys Publischers, Leiden, Margraf Publishers, Weikersheim, 2009, 245-252.
- Op de Beeck, M., De Bock, M., Vandermeiren, K., De Temmerman, L., Ceulemans, R. 2010. A comparison of two stomatal conductance models for ozone flux modelling using data flux modelling using data from two *Brassica* species. Environmental Pollution 158, 3251-3260.
- De Bock, M., Op de Beeck, M., De Temmerman, L., Guisez, Y., Ceulemans, R., Vandermeiren, K., 2011. Ozone dose-response relationships for spring oilseed rape and broccoli. Atmospheric Environment 45, 1759-1765.
- De Bock, M., Ceulemans, R., Horemans, N., Guisez, Y., Vandermeiren K. (submitted) Impact of rising tropospheric ozone on leaf physiology and crop growth in *Brassica napus* L. and *Brassica oleracea* cv Italica
- Vandermeiren, K., De Bock, M., Horemans, N., Guisez, Y, Ceulemans, R., De Temmerman, L. (submitted). Ozone effects on yield quality of spring oilseed rape and broccoli.

6.2 Others

- Vandermeiren K. and De Temmerman L., 2009. Impact of tropospheric ozone pollution on food and feed quality of *Brassica* species. In: Scientific Report 2007/2008, Veterinary and Agrochemical Research Centre. Ed. P. Kerkhofs, Brussels, Belgium. p 94-98.
- Vandermeiren K., De Temmerman L., De Bock M., Op de Beeck M. (2011). Will predicted increases of ozone pollution have an effect on *Brassica* crops? Results of the OFFQ project. In: Scientific Report 2009/2010, Veterinary and Agrochemical Research Centre. Ed. P. Kerkhofs, Brussels, Belgium

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