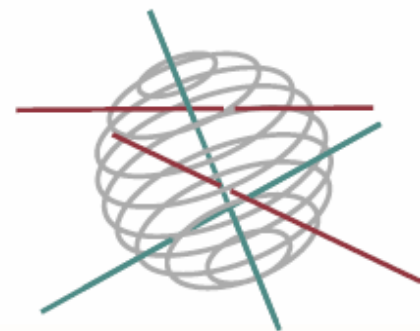


# SSD

SCIENCE FOR A SUSTAINABLE DEVELOPMENT



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

“OFFQ”

K. VANDERMEIREN, M. DE BOCK, N. HOREMANS, M. OP DE BEECK  
R. CEULEMANS, Y. GUISEZ, L. DE TEMMERMAN



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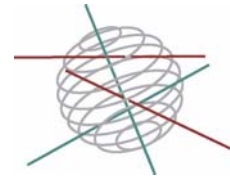
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SCIENCE FOR A SUSTAINABLE DEVELOPMENT  
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***Agro-Food***

FINAL REPORT PHASE 1

**IMPACT OF TROPOSPHERIC OZONE ON FOOD AND FEED  
QUALITY OF BRASSICA SPECIES  
"OFFQ"**

SD/AF/02A

Karine Vandermeiren (VAR)  
Maarten De Bock (UA)  
Nele Horemans (UA)  
Maarten Op de Beeck (UA)  
Reinhart Ceulemans (UA)  
Yves Guisez (UA)  
Ludwig De Temmerman (VAR)

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**VAR**





Wetenschapsstraat 8  
B-1000 Brussels  
Belgium  
Tel: +32 (0)2 238 34 11 – Fax: +32 (0)2 230 59 12  
<http://www.belspo.be>

Contact person: Christine Mathieu  
+32 (0)2 238 34 93

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## CONTACT INFORMATION

### COORDINATOR:

Karine Vandermeiren  
Veterinary and Agrochemical Research Centre (VAR)  
Leuvensesteenweg 17  
B-3080 Tervuren  
Tel: 0032-(0)2-769.22.33  
Fax: 0032-(0)2-769.23.05  
Email: kavan@var.fgov.be

### OTHER PARTNERS:

- Yves Guisez  
University Antwerp, Research Group of Plant Physiology  
Campus Groenenborger, Department of Biology  
Groenenborgerlaan 171  
2020 Antwerpen, Belgium  
Tel: 0032 -(0)3-265.34.15  
Fax: 0032 -(0)3-264.34.17  
yves.guisez@ua.ac.be
  
- Reinhart Ceulemans  
University Antwerp, Research Group of Plant and Vegetation Ecology  
Campus Drie Eiken, Department of Biology  
Universiteitsplein 1  
2160 Wilrijk, Belgium  
Tel: 0032-(0)3-820.22.56  
Fax: 0032-(0)3-820.22.71  
Email: reinhart.ceulemans@ua.ac.be

### PROJECT WEBSITE(S):

[http://www.belspo.be/belspo/ssd/science/projects/OFFQ\\_en.pdf](http://www.belspo.be/belspo/ssd/science/projects/OFFQ_en.pdf)

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## Acronyms, abbreviations and units

APPO	Association pour la Promotion des Protéagineux et des Oléagineux
$A_{sat}$	Light saturated $CO_2$ assimilation ( $\mu mol CO_2 m^{-2} s^{-1}$ )
ASC	Ascorbate
AOT40	Accumulated Ozone exposure over a Threshold of 40 ppb during daylight hours (ppm.h)
C	Coordinator (VAR)
CUO	Accumulated ozone uptake ( $mmol m^{-2}$ )
DHA	Dehydro ascorbate
DW	Dry weight
FW	Fresh weight
$g_s$	Stomatal conductance ( $mol H_2O m^{-2} s^{-1}$ )
$g_{stO_3}$	Stomatal conductance to $O_3$ ( $mol O_3 m^{-2} s^{-1}$ )
GSH	Glutathion
GSL	Glucosinolates
LAI	Leaf area index ( $m^2 leaf/m^2 ground area$ )
NF	Unfiltered air treatment
NF <sup>+</sup>	Unfiltered air treatment + 20 ppb $O_3$
NF <sup>++</sup>	Unfiltered air treatment + 40 ppb $O_3$
NIR	Near Infra Red
OP	Open field plot
OTC	OpenTop Chamber
$O_3$	Ozone
PAR	Photosynthetic Active Radiation ( $\mu mol m^{-2} s^{-1}$ )
P2	Partner 2 (PPG)
P3	Partner 3 (PVE)
PPG	Research Group of Plant Physiology (partner 2)
PVE	Research Group of Plant and Vegetation Ecology (partner 3)
ROS	Reactive Oxygen Species
TAC	Total Antioxidative Capacity
TOC	$\alpha$ -tocopherol
VAR	Veterinary and Agrochemical Research Centre (partner 1)
VPD	Vapour pressure deficit (kPa)

## 1 Summary

The preliminary screening of O<sub>3</sub> sensitivity of four cultivars of *Brassica napus* L. and *Brassica oleracea* L. cv *Italica* during two short term fumigation experiments, was mainly destined to determine the cultivar 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. Despite the limited number of replications the following conclusions could be drawn:

- Acute O<sub>3</sub> exposure caused a reduction of the ASC concentration in broccoli leaves, but without significant differences between cultivars. The GSH concentration was not affected in either plant species.
- Oilseed rape leaves showed a significant increase in gluconapin and glucobrassicinapin in response to O<sub>3</sub>. There was however no significant change in total GSL content for either species.
- 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 replications would be needed to obtain reliable results, especially under less stable conditions such as the OTCs.
- The acute O<sub>3</sub> exposure caused a decrease of photosynthetic activity and chlorophyll concentration, but this effect was only significant for a limited number of cultivars.
- Cultivars Ability (spring oilseed rape) and Monaco (broccoli) were selected for the main OTC experiment because they showed the most significant physiological O<sub>3</sub> response

The short term acute O<sub>3</sub> fumigation has been repeated in 2008 with only one cultivar per species (Ability and Monaco) and the plants were exposed to more elevated O<sub>3</sub> concentrations to obtain significant data on their physiological, biochemical & molecular responses to an acute O<sub>3</sub> exposure. Contrary to previous experiment, visible leaf injury was now indeed observed on *Brassica napus* leaves. The number of physiological assessments were limited, but the chlorophyll fluorescence measurements clearly indicated a comparable decrease in photosynthetic efficiency for both species. The number of active photosynthetic reaction centers per leaf cross section, performance index and potential photosynthetic activity of photosystem II was reduced (significantly for broccoli), whereas energy dissipation showed a tendency to increase. The biochemical analyses from this experiment have not been completely finished yet. This acute O<sub>3</sub> exposure caused a significant increase in total glucosinolate concentration of broccoli leaves, an effect that was not observed during the previous closed chamber experiment.

The Open-Top Chamber experiment was destined to test the effect of a long term, chronic increase in O<sub>3</sub> concentrations on the yield quantity and quality of *Brassica napus* and *Brassica oleracea* cv *Italica* under near field conditions. The main interest was to investigate whether a further increase in ambient O<sub>3</sub> pollution may induce changes in glucosinolate and vitamin content of the consumable end products which is important for the health and safety of the food and feed chain.

There was an important difference in growth conditions and O<sub>3</sub> exposure levels between 2007 and 2008, especially for *Brassica napus*. In 2007 emergence of oilseed

rape was not optimal due to a serious drought that lasted for three weeks. In the OTCs plant density was more seriously reduced in comparison to the OPs (60 vs. 90 plants/m<sup>2</sup>) mainly because the constant airflow caused a higher water evaporation of the soil. Especially the NF treatment yielded some conflicting results due to the irregular plant emergence in 2007. For broccoli the results of both growing seasons were much more comparable and most of the data could be pooled for statistical analysis. In 2008 the O<sub>3</sub> exposure level was much more elevated because the ambient concentrations were higher in comparison to 2007. As a consequence, in 2008 visible leaf injury was observed on both species in the most elevated O<sub>3</sub> treatments, which was not the case in 2007. Whereas the bad weather conditions of 2007 limited the number of field measurements, in 2008 crop growth and its physiological evolution could be perfectly monitored throughout the growing season. It was also decided, as recommended by the follow-up committee, to put more effort into the OTC assessment and reduce some of the OP measurements in 2008.

Exposure to an increased level of O<sub>3</sub> pollution caused a number of physiological changes in oilseed rape crops, that were mainly related to increased senescence. This was apparent after flowering and resulted in a more rapid loss of leaf area, decrease of photosynthetic activity ( $A_{\text{sat}}$ ) and of photochemical efficiency assessed by several chlorophyll fluorescence techniques (potential and effective photochemical efficiency, performance index, photochemical reaction centers per leaf cross section, energy fluxes through photosystem II). This was accompanied by an increased loss of energy at the level of the antenna chlorophylls. At the leaf level the appearance of these effects depend on the leaf's phenological age. Chlorophyll measurements on younger, upper canopy and older, lower canopy leaves show that the same process takes place in all the leaves, but the effects become more significant as the leaves grow older.

This explains why the light saturated CO<sub>2</sub> assimilation and Fv/Fm of upper canopy leaves in broccoli are not significantly affected by O<sub>3</sub>. These leaves remain green and healthy during the entire growing season. At harvest the young green broccoli florets are harvested before plants are allowed to senescence and produce seeds. However, the limited number of measurements on lower canopy leaves do reveal a decrease of photosynthesis in response to higher O<sub>3</sub> concentrations. Fluorescence measurements after dark adaptation also seem slightly more sensitive to detect decreases in photosynthetic efficiency.

At the moment, biochemical and molecular analyses at the leaf level have only been partly completed. Data from 2007 give some indications of an increased antioxidant capacity in response to O<sub>3</sub>. In both years the  $\alpha$ -tocopherol concentration of broccoli leaves, sampled during the generative phase, was reduced by O<sub>3</sub>. In oilseed rape leaves there is a tendency for total and reduced ascorbate to be increased by O<sub>3</sub> during the generative phase whereas the opposite tendency appears for broccoli. The latter was also observed after the acute O<sub>3</sub> fumigation.

As indicated, the irregular emergence of oilseed rape in 2007 poses some problems, especially for the interpretation of the yield effects, both on quantity and quality. 2008 offers a much higher degree of reliability. The highest level of O<sub>3</sub> exposure induced a 29 % decrease of seed yield in 2008. Though not significantly, the same degree of reduction was achieved in 2007, when comparing NF<sup>++</sup> to the intermediate O<sub>3</sub> exposure level (NF<sup>+</sup>). This was correlated to a decrease of the 1000 seed weight (-14 and -9%, not significant), but there were no changes in seed density (weight/volume). Lipids and proteins are quantitatively the two most important fractions of oilseed rape seeds and account for more than 60% of the seed weight.



The protein content of the seeds was not affected by O<sub>3</sub>. In 2008, O<sub>3</sub> induced a clear decrease of oleic acid and of the oil %, accompanied with an increase in linoleic acid and % saturated fatty acids. The same tendency appears to be present for the NF<sup>++</sup> compared to NF<sup>+</sup> treatments in 2007. The increase of the linoleic acid content is important with regard to human nutrition because it is an omega 6 fatty acid that we cannot synthesize ourselves. In animal feed, glucosinolates may decrease digestibility and cause goitre and haemolytic anaemia if supplemented at excessive rates. In this respect it is important that the glucosinolate concentration of rapeseed meal does not exceed certain safety limits. The seeds from our experiments mainly contained progoitrine, gluconapin and 4OH-glucobrassicin. O<sub>3</sub> did not cause a significant change in total glucosinolate concentration. Neither did we detect a significant shift in the relative GSL composition.

Fresh marketable yield of broccoli vegetables was significantly reduced by elevated O<sub>3</sub> in 2007, but not in 2008, despite the higher O<sub>3</sub> exposure level. This was also reflected in the dry weight of the individual plant parts and total aboveground plant weight. The reason for this discrepancy is not clear yet. Perhaps the earlier harvest in 2008 had an influence, or the differences in O<sub>3</sub> uptake (flux) may provide an explanation. A preliminary parameterisation of the stomatal conductance for O<sub>3</sub> allowed a first estimate of the actual O<sub>3</sub> uptake in comparison to the O<sub>3</sub> concentrations. This already indicates a large variation in O<sub>3</sub> uptake depending on climatic conditions. Moreover, in comparison to the AOT40, broccoli shows a relatively higher O<sub>3</sub> uptake than oilseed rape. These data surely need further investigation to determine O<sub>3</sub> uptake-response relationships for application within O<sub>3</sub> risk assessments.

The quality of broccoli vegetables in relation to human nutrition is determined by their protein, vitamin and glucosinolate content. The protein content of broccoli plant parts was not significantly affected by an increase in O<sub>3</sub> exposure in 2007 and 2008. The data from 2007 did not indicate a change in vitamin content either, but the data of 2008 are not yet available. The long term increase in O<sub>3</sub> concentration caused a significant increase of the aliphatic GSLs glucoiberin and glucoraphanin, the latter being the most abundant GSL in broccoli vegetables. This did not, however, result in a significant increase of the total GSL content as the effect seemed to be counteracted by a (not significant) decrease of the indole GSLs glucobrassicin and neo-glucobrassicin. This is an important shift since it is mainly the ratio aliphatic/indole GSLs that is important with regard to human health. The anticarcinogenic properties of broccoli are attributed to the aliphatic compounds such as glucoraphanin, the indole GSLs on the contrary are often considered carcinogenic.

In summary we may state that the first phase of the OFFQ project has been executed according to the time schedule of the proposal, except for the molecular analyses that have experienced some delay. Thanks to the input of the follow-up committee and additional staff, this problem is now solved and this information will become available in 2009. The results obtained until now clearly confirm that increased ambient O<sub>3</sub> pollution will induce physiological and biochemical changes for the investigated *Brassica* crops. Whether the effects on yield quality will have a significant impact on the health and safety aspects of the food and feed chain still needs to be further investigated. The challenge during the second phase will also be to relate these physiological and biochemical effect to the changes at the molecular level.

## 2 Context

Ozone (O<sub>3</sub>) 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<sub>3</sub> protects the Earth's surface from solar UV radiation, tropospheric O<sub>3</sub> is (after CO<sub>2</sub> and CH<sub>4</sub>) the third most important greenhouse gas (Denman et al, 2007; Solomon et al, 2007). It contributes to greenhouse radiative forcing causing a change in the balance between incoming solar radiation and outgoing infrared radiation within the atmosphere that controls the Earth's surface temperature. Besides its role as a direct greenhouse gas, O<sub>3</sub> has been identified as one of the major phytotoxic air pollutants, affecting human health and materials, as well as vegetation (WGE, 2004).

Increased emissions associated with fossil fuel and biomass burning (Gauss et al, 2006; Denman et al, 2007), long-distance and even intercontinental transport have resulted in a steady increase in O<sub>3</sub> concentration in rural areas hundreds and thousands of kilometres from the original sources of pollution (Prather et al, 2003). Simulations for the period 2015 through 2050 even predict further increases in tropospheric O<sub>3</sub> of 20 to 25% (Meehl et al, 2007). The global patterns of exposure of vegetation to O<sub>3</sub> are also changing. Control measures on emissions of nitrogen oxides (NO<sub>x</sub>) and volatile organic compounds (VOCs) applied in North America and western Europe, where the impacts of O<sub>3</sub> on crop production and forest vitality have been well established, are expected to lead to reductions in peak O<sub>3</sub> concentrations (e.g. Gardner & Dorling, 2000). However, the effect of these changes may be offset by the predicted increases in global background tropospheric concentrations, in particular as a result of increased global emissions of NO<sub>x</sub> (NEG-TAP, 2001).

There is no doubt that these predicted increases in tropospheric O<sub>3</sub> will impact on future agro-ecosystems and their management. O<sub>3</sub> 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<sub>3</sub> itself can modify the response of plants to a range of naturally occurring environmental stresses such as drought (Bell, 1987). This explains why O<sub>3</sub> has been recognized as an excellent tool to study signalling cascades that involve apoplastic ROS formation in the regulation of gene expression. As such, it can be used to increase 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 (Fenwick et al, 1989). Most importantly, these biochemicals have been attributed anticarcinogenic properties. 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 ( $\alpha$ -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, this study aims to contribute to the risk assessment of the impact of predicted increases in tropospheric O<sub>3</sub> on changes in yield, quality and safety of *Brassica* species as primary source for human nutrition and animal feed and 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 stabilise 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.

### 3 Objectives

1. To determine the impact of increasing tropospheric O<sub>3</sub> concentrations on antioxidant (ascorbate (ASC), glutathione (GSH), α-tocopherol (TOC), total antioxidant capacity (TAC)) and glucosinolate (GSL) composition of *Brassica* species. These compounds are highly important in relation to the health and safety aspects of human food and animal feed. This will be achieved by exposure of oilseed rape and broccoli to different O<sub>3</sub> levels, both as either acute peak episodes or under “near-field” conditions and by subsequent analysis of the consumable end product.
2. To evaluate the influence of O<sub>3</sub> on the human diet and animal feed intake by incorporating the changes in antioxidant and GSL levels of the consumable end products (broccoli and rape seed meal) in the food and feed chain
3. To identify physiological and biochemical biomarkers for O<sub>3</sub> stress by investigating the interaction between stress induction and changes in secondary metabolites. Therefore physiological assessments of plant stress responses (photosynthesis and chlorophyll fluorescence) will be linked to biochemical analysis of antioxidants and GSLs at the leaf level.
4. To elucidate the interaction between abiotic stress induction, defence pathways and changes in secondary metabolites by means of transcriptomic analysis
5. To evaluate the impact of O<sub>3</sub> induced changes in GSL content and composition at the leaf level in relation to plant-pathogen/insect interaction through literature study
6. To determine yield losses and changes in yield quality by comparison of plant production under different levels of O<sub>3</sub> exposure in Open-Top Chambers
7. To contribute to O<sub>3</sub> flux modelling by providing data on environmental dependence of stomatal conductance of oilseed rape and broccoli. This will lead to O<sub>3</sub> uptake - response functions for O<sub>3</sub> risk assessment on vegetation.

## 4 Materials & Methods

For detailed description of methodology see also technical specifications of Contract n° SD/AF/02A. The tasks have been performed by either the coordinator (C), partner 2 (P2) or partner 3 (P3) as indicated in the text.

### 4.1 Plant material

The choice for the *Brassica* species oilseed rape or canola (*Brassica napus* L.) and broccoli (*Brassica oleracea* L. cv. Italica) is 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. *Brassica napus* is considered rape when its seed contains high concentrations of glucosinolates and erucic acid. *B. napus* varieties with lower quantities of these compounds are called “double zero” (EU) or Canola (Canada) (OECD, 2000).
- Health: *Brassica* vegetables contain vitamins and glucosinolates that have potential value as cancer chemopreventive 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 (Orthofer, 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 John Innes Centre in the UK has selected a broccoli variety with increased glucosinolate content, and the seed company Seminis aims to further develop this for better adaptation to a variety of different climates.
- 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.

### 4.2 Short acute O<sub>3</sub> exposure in environmentally controlled chambers (C)

Initially, in 2007, two short term, acute O<sub>3</sub> fumigations have been performed in 4 environmentally controlled chambers ( 1.5 x 2.2 x 2.4 m) located at VAR. These experiments were 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 was determined and measuring techniques for photosynthesis and fluorescence assessments were tested. The physiological measurements were destined to identify differences in O<sub>3</sub> sensitivity between the cultivars. Each chamber contained 4 commercial cultivars of broccoli (*Brassica oleracea* L. cv Italica) and oilseed rape (*Brassica napus* L.), cultivated in pots, 3 plants per cultivar. The selection of these cultivars was mainly based on their relative importance for the Belgian market. All oilseed cultivars were 00 varieties with low erucic acid and GSL content, suitable for human and animal consumption. For oilseed rape 2 spring cultivars (Ability & Simon) and 2 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<sub>3</sub> 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<sub>3</sub> exposure (table 4.1).

Plants were approximately 6 weeks old at the time of fumigation. Slow release fertiliser (Osmocote 15/10/12 + Mg + micro-elements) was added to the pot soil before seedling transfer.

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<sup>-2</sup> s<sup>-1</sup> 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<sub>2</sub>, NO, NO<sub>2</sub> and O<sub>3</sub> concentrations were reduced to < 15 ppb. In two other chambers, O<sub>3</sub> was added at the desired concentrations during 5h/day by means of mass flow controllers. O<sub>3</sub> was generated by electrical discharge from pure oxygen with an ozone generator (CMG 3-3, Innovatec, The Netherlands). O<sub>3</sub> concentrations were continuously monitored by UV absorption (41M, Environnement S.A, France); NO<sub>x</sub> concentrations were measured by chemiluminescence (model 200A, API, USA).

Table 4.1: Overview of the fumigation experiments in closed chambers at VAR

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

### 4.3 Long term chronic O<sub>3</sub> exposure in Open-Top Chambers (OTCs) (C)

In 2007 & 2008 oilseed rape and broccoli crops have been exposed to different levels of O<sub>3</sub> for 8 hrs/day during their entire growth, from sowing or planting until harvest. The exposure was performed in 15 open-top chambers. These chambers have a decagonal ground plan (3 m diameter); the soil surface covers 7.3 m<sup>2</sup> and the chamber volume is 19.6 m<sup>3</sup>. 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 4 air changes are accomplished at a flow rate of 1.36 m<sup>3</sup> s<sup>-1</sup>, 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. Six unchambered field plots (OPs) were included in the experimental set-up to compare the micro climatic conditions and differences in plant growth between OTCs and ambient air (chamber effect). Moreover, these plots were used to perform measurements of stomatal conductance that provide data for the ozone flux modelling needed for further developing the ozone critical levels for vegetation as requested by the Convention on Long-Range Transboundary Air Pollution (LRTAP, 2004).

The supply of O<sub>3</sub> is adjusted with mass flow controllers controlled by a microprocessor in reference to the actually measured O<sub>3</sub> concentrations. O<sub>3</sub> is produced from 10 until 18h GMT with an O<sub>3</sub> generator (Fischer OZ500, Germany) from pure oxygen by electric discharge (Vandermeiren, 2003). 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.

Before sowing or planting the plots were fertilized according to the recommendations of the “Bodemkundige Dienst van België” based on previous soil analysis (table 4.2).

Table 4.2: Fertilizer quantity applied in OTCs and OPs prior to sowing/planting (kg/ha)

Fertilizer	Oilseed rape		Broccoli	
	OTC	OP	OTC	OP
Ammonium sulphate (21% N)	57	58	98	106
Triple phosphate (45% P <sub>2</sub> O <sub>5</sub> )	22	17	36	33
Patent potassium (30% K <sub>2</sub> O)	80	82	103	110
Lime (48 acid binding value)	0	210	198	188
Na (52% Na <sub>2</sub> O)	0	7	3	10
Borax (11% B)	0.7	0.7	0.7	0.7

*Brassica napus* cv Ability was sown in 9 OTCs and 3 OPs (3 blocks each containing an unfiltered air treatment (NF), two O<sub>3</sub> treatments NF+20 ppb O<sub>3</sub> (NF<sup>+</sup>) & NF+40 ppb O<sub>3</sub> (NF<sup>++</sup>) and one OP. *Brassica oleracea* L. cv Monaco was sown and precultivated in a climatically controlled chamber and greenhouse. After 5-6 weeks the plants were transferred to 6 OTCs and 3 OPs (3 blocks each containing an NF & NF<sup>++</sup> treatment & one OP) at 50 cm spacing within and between the rows (for details see table 4.3). 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 or 7 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 analysis at VAR. For broccoli 12 plants per chamber were harvested. Dry material was ground and stored for further analysis.

Table 4.3: Overview of crop cultivation, treatments and yield quantity + quality data in 2007& 2008

	<i>Brassica napus</i> cv Ability	<i>Brassica oleracea</i> cv Monaco
Sowing date	sown in full soil 18/04/2007 10-11/04/2008	sown in climate chamber 02/05/2007 07/05/2008
50 % emergence /planting date	09/05/2007 21/04/2008	13/06/2007 04/06/2008
Plant density	120 pl/m <sup>2</sup>	4 pl/m <sup>2</sup>
Intermediate harvest	Not in 2007 20/05/2008	No intermediate harvest
Harvest	21/08/2007 (7 subplots/chamber) 08/08/2008 (6 subplots/chamber)	07/08/2007 (12 plants/chamber) 5-6/08/2008 (12 plants/chamber)
Yield quantity	Seed yield, 1000 seed weight, seed volume/weight	FW + DW of leaves, stems & inflorescence
Yield quality	Seeds : •Fatty acids •Glucosinolates •Proteins •Vitamins	Florets •Glucosinolates •Proteins •vitamins

O <sub>3</sub> fumigation	25/05/2007 - 10/08/2007 07/05/2008 – 08/08/2008	13/06/2007 - 07/08/2007 09/06/2008 – 06/08/2008
treatments	3 NF = unfiltered air 3 NF <sup>+</sup> = NF + 20 ppb O <sub>3</sub> 3 NF <sup>++</sup> = NF + 40 ppb O <sub>3</sub> 3 OP = open field plots	3 NF = unfiltered air No NF <sup>+</sup> 3 NF <sup>++</sup> = NF + 40 ppb O <sub>3</sub> 3 OP = open field plots

#### 4.4 Physiological assessments (C & P3)

At the end of the closed chamber experiment and throughout crop growth in the OTC experiments, physiological assessments of plant performance have been carried out by the Research Group of Plant and Vegetation Ecology, department of Biology, University of Antwerp. The main objective of these measurements was to identify the extent to which O<sub>3</sub> was causing a physiological stress response and to relate these events to changes in biochemical profiles. 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.

Following measurements have been performed:

- Light saturated photosynthesis ( $A_{sat}$ ) & stomatal conductance ( $g_s$ ) with LI-6400 (Li-Cor, USA) at 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  Photosynthetic Active Radiation (PAR)
- Chlorophyll fluorescence: actual photochemical efficiency ( $F'v/F'm$ ) with LI-6400 (Li-Cor, USA) or potential photochemical efficiency ( $Fv/Fm$ ) and Performance Index (PI) with Handy Pea (Hansatech, UK).
- Fluorescence imaging with a prototype of a portable chl fluorescence imaging system suitable for field measurements (Gielen *et al.*, 2006).
- 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 according to the method of Wellburn and Lichtenthaler (1984).
- Leaf area index (LAI) with the LAI-2000 Plant Canopy Analyser (Li-Cor, USA)

#### 4.5 Glucosinolate analysis (C)

Fresh plant material (at least 10 g/sample) was immediately frozen in liquid nitrogen, stored at -80°C and the extraction was performed as soon as possible. For oilseed samples, 200 mg of air dried material was used. Analysis is performed according to the (slightly modified) International Standard ISO 9167-1 (1992). Frozen tissues (1g) are ground with liquid nitrogen, extracted with methanol at 70-75°C and an internal standard (2  $\mu\text{mol}$  sinigrine or glucotropaeoline) is added. After centrifugation the supernatant is dried in a rotavapor, resuspended with distilled water and filtered. The extract is transferred to an ion-exchange column of DEAE Sephadex A25, activated with imidazol formate and pH is adjusted with sodium acetate buffer (pH 4). The extract is desulfated overnight with a sulfatase solution of *Helix Pomata* and subsequently the desulfoglucosinolates are eluted with water. The glucosinolates are separated by means of HPLC (Alliance 2695, Waters, USA) over a C18 column (Atlantis<sup>TM</sup> dC18, 3  $\mu\text{m}$ , 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  $\mu\text{mol/g}$  air dried material for oilseeds (<10% moisture) and fresh or oven dried weight for all other plant tissues.

#### **4.6 Ascorbate (ASC) , glutathione (GSH) and $\alpha$ -tocopherol (TOC) analysis (P2)**

For all biochemical analyses described below, fresh plant material (+/-100mg) was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until extraction. For determination of ASC en GSH-content, first the samples are grounded using glass beads and a Magnalyzer (Roche applied science). At this stage it is essential to keep samples frozen to prevent oxidation. Subsequently the antioxidants are extracted in an acidic environment by addition of ice-cold meta-phosphoric acid (6%, 1ml/200 mg of sample). The samples are clarified by centrifugation at 16 000g at  $4^{\circ}\text{C}$  for 20 minutes. Antioxidants are separated on a 100 mm x 4.6 mm Polaris C18-A reversed phase HPLC column (3  $\mu\text{m}$  particle size;  $30^{\circ}\text{C}$ ; Varian, CA USA) with an isocratic flow of 1 ml/min of the elution buffer (25 mM  $\text{KPO}_4$ -buffer, pH 3.00). The components are quantified using a homemade electrochemical detector with glassy carbon electrode and a Schott pt 62 reference electrode (Mainz, Germany). The purity and identity of the peaks is confirmed using a diode array detector (SPD-M10AVP, Shimadzu, Hertogenbosch, Netherlands), which is placed online with the electrochemical detector. The amount of oxidised DHA or GSH concentration is measured indirectly as the difference between the total concentration of antioxidants in a DTT reduced fraction and the concentration in the sample prior to reduction. Reduction of the sample is obtained by incubation of an aliquot of the extract in 400 mM Tris and 200 mM DTT for 15 min in the dark.

For  $\alpha$ -TOC determination an organic extract is made. Samples are again grounded using glass beads and Magnalyzer (Roche). Then  $\alpha$ -TOC is added as internal standard (100  $\mu\text{l}$ , 20  $\mu\text{g/l}$ ) together with 1 ml of hexane and samples are intensely shaken using the Magnalyzer device. After brief centrifugation eluens is collected, subsequently the samples are re-extracted using again 1 ml of hexane, in total 4 ml of hexane is used to extract all  $\alpha$ -TOC. The combined fraction is subsequently filtered through a pvdf filter (0,45  $\mu\text{m}$ ), concentrated under vacuum, and separated on a 250 mm x 4.6 mm Partical PAC 5u HPLC column (5  $\mu\text{m}$  particle size;  $40^{\circ}\text{C}$ ; Alltech, IL USA) with an isocratic flow of 1,5 ml/min of the elution buffer (hexane, 8% Tetrahydrofuran). The concentration of  $\alpha$ -TOC is determined with the fluodetector (ex 290 nm, em 330 nm, RF10, Shimadzu, Hertogenbosch, Netherlands) after comparison with the internal standard. The purity and identity of the peaks is confirmed using a diode array detector (SPD-M10AVP, Shimadzu, Hertogenbosch, Netherlands), placed on line with the fluodetector.

#### **4.7 Total antioxidative capacity (TAC) (P2)**

Samples are grounded using the Magnalyzer (Roche) as described for ASC en GSH. They are kept frozen until addition of the acidic extraction medium (0,01 N HCl) and afterwards treated below  $4^{\circ}\text{C}$ . Samples are clarified by centrifugation at 16000g for 20 minutes. TAC was measured as the capacity of antioxidants to reduce the complex  $\text{Fe}^{3+}$ -TBTZ to  $\text{Fe}^{2+}$ -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  $\mu\text{M}$  and therefore TAC was expressed as  $\mu\text{mol}$  TROLOX equivalents/g FW.

#### **4.8 Transcriptomic analysis (P2)**

To examine a large amount of gene-expressions, a microarray (*Brassica* Expression Profiling Service) is being performed by Cogenics. Afterwards expression levels of most interesting genes will be additionally confirmed using quantification with real-time PCR technique. Real time methods have the advantage that the amount of cDNA is measured after each duplicating cycle. The amount of starting concentration of one specific (amplified) gene can be determined very accurately (Gachon *et al*, 2004). RNA extraction was performed using the Concert Plant RNA reagent. 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 describer. Quantity of RNA was determined spectrophotometrically (Nanodrop, Nanodrop technologies), quality was controlled using a gel electrophoresis separation method (Qiagxcel, Qiagen). Total RNA was reversed transcribed to first strand cDNA using Superscript III Reverse transcriptase kit (Roche). Quality good cDNA was combined from different replicates and sent out to Cogenics (UK) for microarray analyses. The actual real-time PCR reaction will be 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 enable quantification. Afterwards calculation of the expression levels compared to house-keeping genes will be done as described earlier (Pfaffl, 2001).

#### **4.9 Fatty acid analysis (C)**

Fatty acid analysis of oilseeds has been 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.

#### **4.10 Protein analysis (C)**

Protein analysis of canola seeds is determined by near infra red analysis at the Centre Wallon de Recherches Agronomiques (Gembloux, Belgium). The crude protein content of all other plant material is calculated by multiplying the nitrogen content by 6.25 (EuropaBio, 2001). Total nitrogen is determined by the Kjeldahl method after digestion with sulphuric acid and addition of Se reagent mixture.

#### **4.11 Ozone flux modelling (P3)**

The gas exchange measurements are used for parameterization and validation of two distinct O<sub>3</sub> flux algorithms. These algorithms simulate oilseed rape and broccoli stomatal O<sub>3</sub> fluxes for all fumigation regimes, as stomatal flux is the physiologically relevant O<sub>3</sub> exposure metric which measured biochemical and ecophysiological plant ozone responses might be linked to. Both algorithms calculate stomatal fluxes from measured O<sub>3</sub> concentrations and simulated stomatal conductance to O<sub>3</sub>.

## 5 Results

### 5.1 Differences in acute O<sub>3</sub> response between cultivars

The aim of the short term fumigation experiments performed in 2007 in the climatically controlled chambers, was mainly to observe whether any clear differences in O<sub>3</sub> sensitivity might exist between broccoli and oilseed rape cultivars. Due to space limitations however, we were only able to screen a limited number of cultivars. Therefore a primary selection was made based on the economic importance of the cultivars for the Belgian market.

#### 5.1.1 Physiological stress responses

The O<sub>3</sub> treatment did not induce any visible injury symptoms. Although there were not many clear statistical differences with regard to the physiological assessments, some preliminary conclusions can be drawn:

- O<sub>3</sub> induced a more pronounced reduction of A<sub>sat</sub> and F'v/F'm and SPAD in spring oilseed rape cultivars Ability and Simon compared to the winter cultivars Grizzly and Hydromel (fig 5.1 a,c,e)
- Ability was the only oilseed rape cultivar that showed a significant decrease of F'v/F'm and SPAD in response to the O<sub>3</sub> fumigation (Fig 5.1 c,e)
- The broccoli cultivars showed a general reduction of F'v/F'm in response to O<sub>3</sub> exposure (Fig 5.1 d)
- Both Lord and Monaco showed a decrease of A<sub>sat</sub> and F'v/F'm in the O<sub>3</sub> compared to the control treatment (Fig 5.1 b,d)

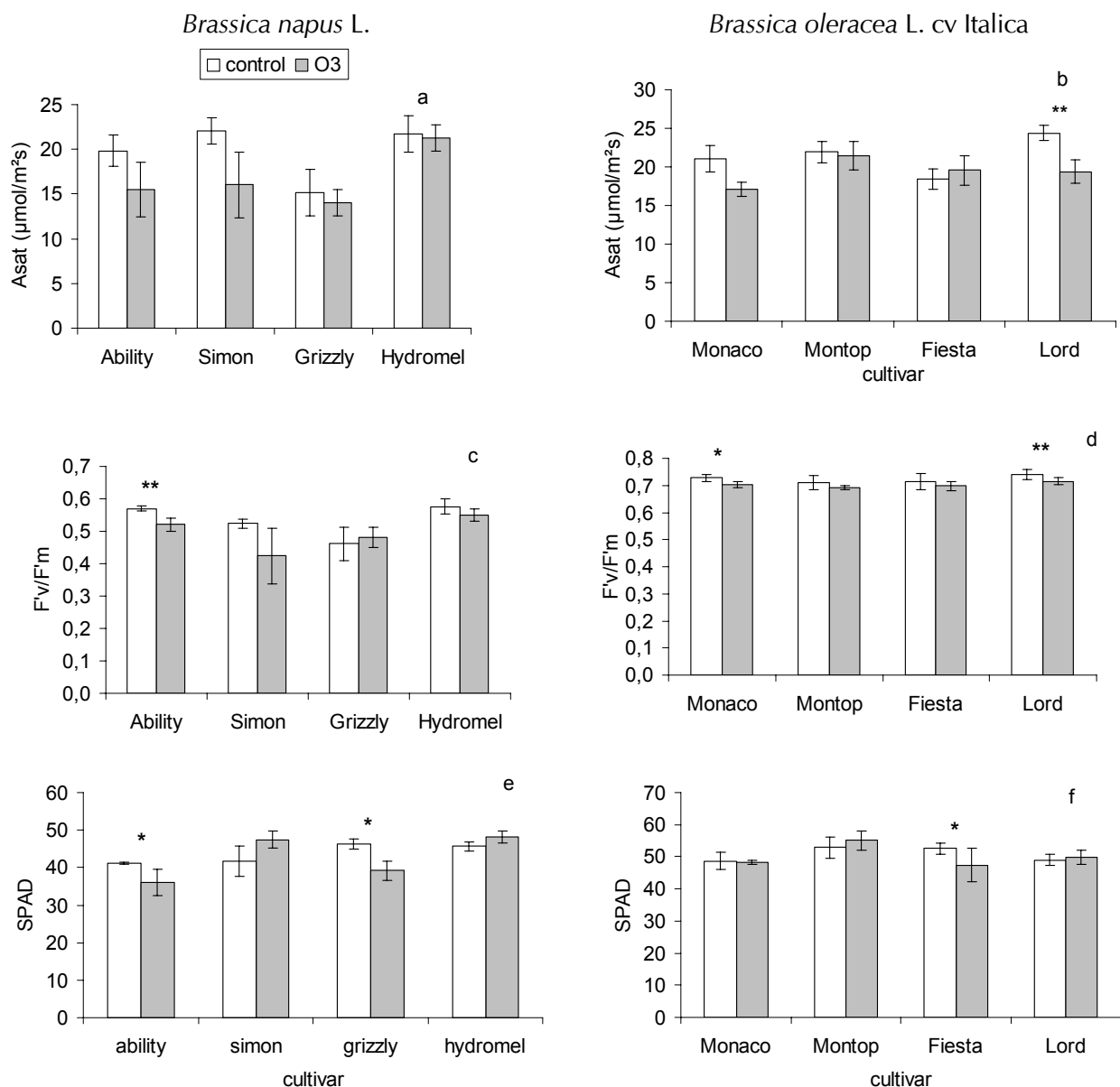


Fig. 5.1:  $\text{O}_3$  effect on light saturated  $\text{CO}_2$  assimilation ( $A_{sat}$ ) (a,b), actual photochemical efficiency ( $F'_v/F'_m$ ) (c,d) and chlorophyll content (SPAD) (e,f) of oilseed rape (a,c,e) and broccoli cultivars (b,d,f). Significance of the difference with NF has been determined by ANOVA ( $0.05 < p \leq 0.1$ )

### 5.1.2 $\text{O}_3$ effect on antioxidants

The ASC, GSH and TAC analyses were completed after the start of the  $\text{O}_3$  experiment and consequently these results could not contribute to the choice for a particular cultivar for the long term  $\text{O}_3$  experiment. Fig 5.2b shows an overall  $\text{O}_3$  induced reduction of vitamin C (ASC) in the leaves of broccoli, although this effect was not statistically significant for any of the individual cultivars. For oilseed rape no changes in ASC or GSH were detected in response to the acute  $\text{O}_3$  treatment, mainly due to a large variation between individual samples.

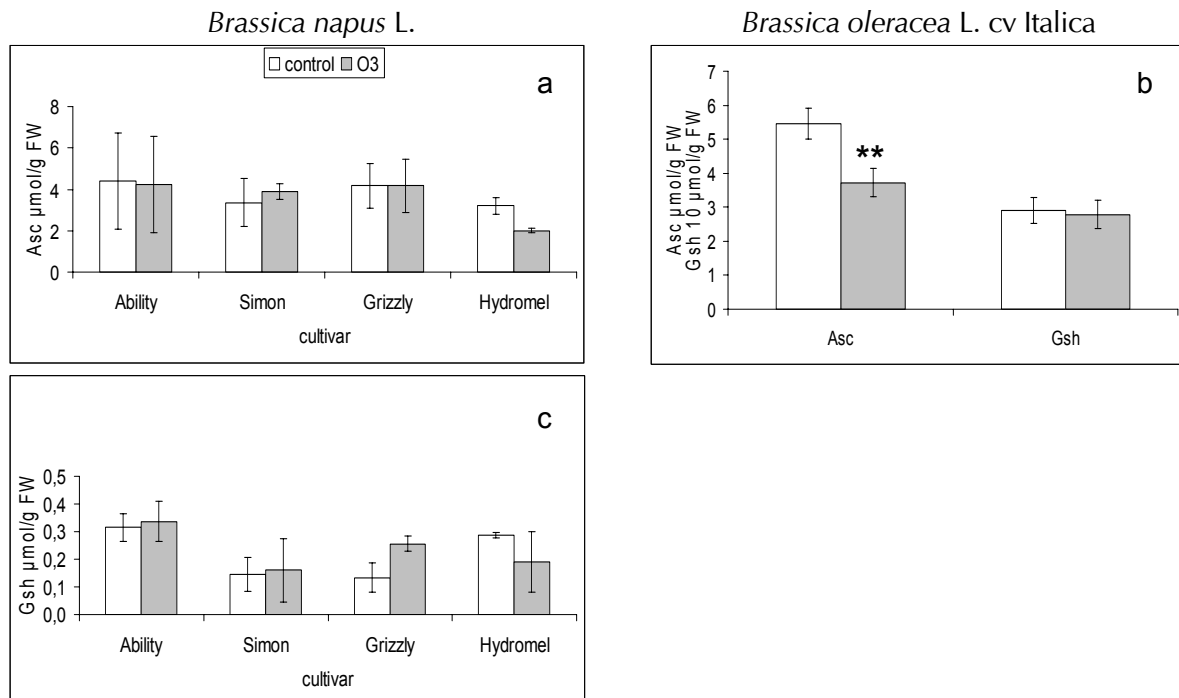


Fig. 5.2: O<sub>3</sub> effects on ascorbate (a,b) and glutathione (b,c) concentration of oilseed rape (a,c) and broccoli (b) Significance of the difference with NF has been determined by 2 sample t test (\*\* 0.01 < p ≤ 0.05)

### 5.1.3 O<sub>3</sub> effect on glucosinolates

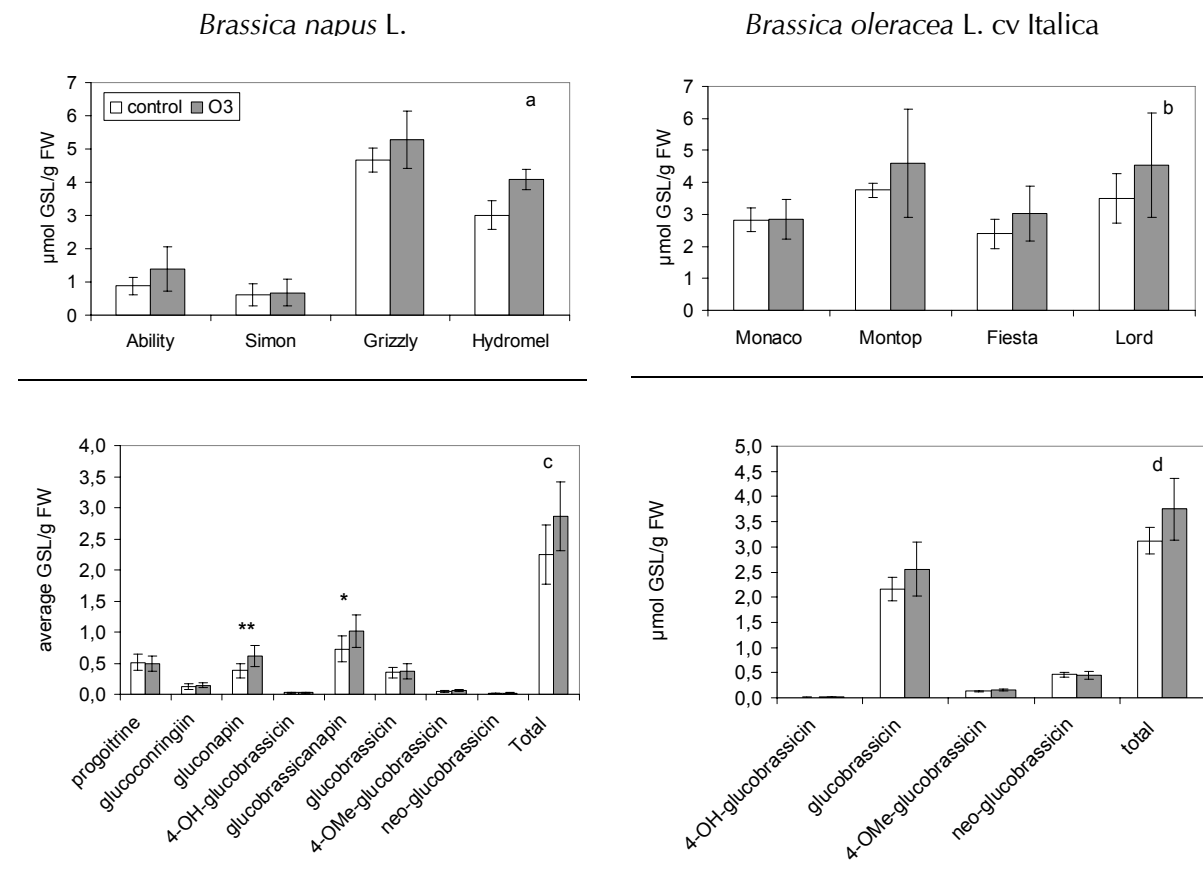


Fig. 5.3: O<sub>3</sub> effects on total GSL content of young leaves of oilseed rape (a) and broccoli (b) cultivars. The concentration of the most important individual GSLs has been averaged over the 4 cultivars of oilseed rape (c) and broccoli (d). Significance of the O<sub>3</sub> effect has been determined by ANOVA (\* 0.05 < p ≤ 0.1; \*\* 0.01 < p ≤ 0.05)

Analytical results of leaf samples originating from 4 cultivars exposed to a short acute O<sub>3</sub> exposure, have been pooled, both for oilseed rape and broccoli. Despite some cultivar differences (winter cultivars of oilseed rape contain higher levels of GSLs compared to spring cultivars - fig 5.3a), no significant cultivar × O<sub>3</sub> interactions have been detected. The youngest, fully developed leaves of oilseed rape mainly contain progoitrine, gluconapin and glucobrassicin. The major GSL in broccoli leaves is glucobrassicin. Although there was a general tendency for total GSLs to increase in response to O<sub>3</sub>, the statistical evidence was not significant. Only gluconapin and glucobrassicin showed a significant increase in colza leaves.

## 5.2 Responses of oilseed rape (cv Ability) and broccoli (cv Monaco) to a short acute O<sub>3</sub> episode

As mentioned previously the closed chamber fumigation experiment has been repeated in April 2008 with only one cultivar of each species and at a slightly higher O<sub>3</sub> level (150 instead of 100 ppb) to increase the statistical significance of the physiological assessments and of the biochemical and molecular analyses. Each

chamber contained 18 plants per cultivar; there were two control chambers with Purafill filtered air and two chambers with an elevated O<sub>3</sub> treatment. All measurements and samples were taken after 5 days of O<sub>3</sub> exposure:

- chlorophyll fluorescence with Handy Pea on 6 plants/species/chamber
- fluorescence imaging on 4 plants/species/chamber
- SPAD measurements on 10 plants/species/chamber
- leaf samples for ASC, GLH, TAC & RNA of 10 plants/species/chamber
- GSL leaf samples of 10 plants/species/chamber

### 5.2.1 Visible O<sub>3</sub> injury

Contrary to the previous experiment, visible injury symptoms were observed on O<sub>3</sub> treated *Brassica napus* (Fig 5.4) and the percentage of flowering plants was increased.



Fig. 5.4: Leaf necrosis and chlorosis on *Brassica napus* caused by elevated O<sub>3</sub>

### 5.2.2 Acute physiological effects

The destructive determination of the leaf chlorophyll content yielded following calibrations curves that can be used to recalculate the SPAD values (Fig 5.5). It became obvious that for broccoli SPAD values above 55 could not be reliably correlated with the true chlorophyll content (fig 5.5 b)

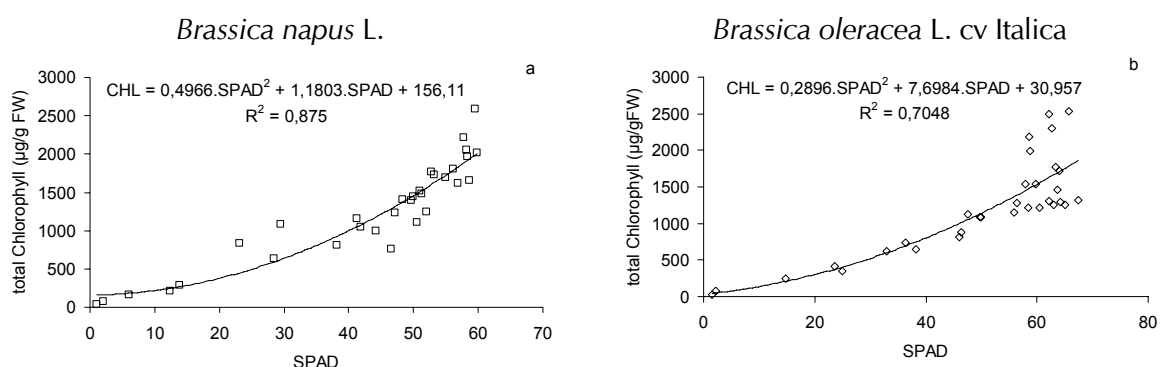
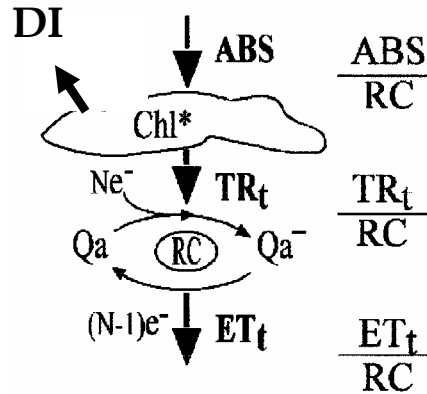


Fig. 5.5: Relationship between the undestructive measurements of chlorophyll concentrations (a+b) of oilseed rape (a) and broccoli leaves (b) with the Minolta SPAD-502 meter and the spectrophotometric assay from extracts of leaf discs.

The efficiency of photosystem II was assessed by chlorophyll fluorescence measurements after dark adaptation. The potential photochemical efficiency is

determined as Fv/Fm. 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<sub>2</sub> 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<sub>3</sub> response with regard to these parameters: the potential photochemical efficiency (Fv/Fm) was slightly decreased, but the performance index (PI) and number of active photochemical reaction centers per leaf cross section showed an even more pronounced decrease (Fig 5.6). 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.

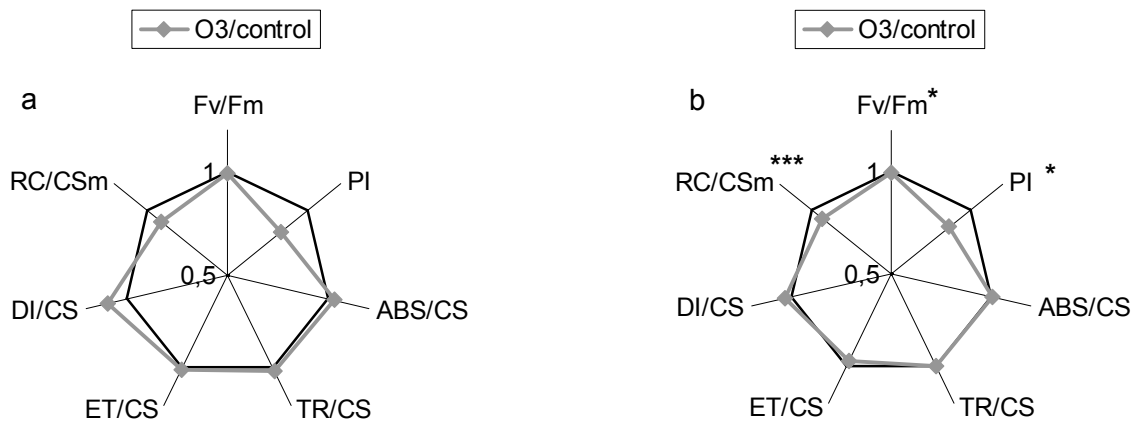


Fig. 5.6: Ratio of fluorescence parameter value of the O<sub>3</sub> treated versus control plants of oilseed rape (a) and broccoli (b). \* and \*\*\* indicate a 90 and 99% sign O<sub>3</sub> effect (GLM)

Fluorescence imaging did not reveal any significant differences between O<sub>3</sub> treated and control plants for either species (Fig 5.7). However, the mathematical and statistical analysis of the images may deserve further investigation (see 5.3.5).



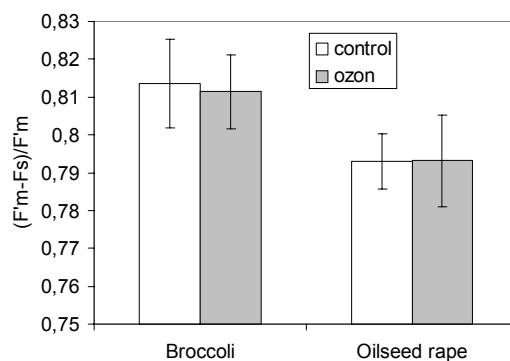


Fig. 5.7:  $O_3$  effect on the actual photochemical efficiency of oilseed rape and broccoli, as determined by fluorescence imaging, was not significant ( $p>0.10$ )

### 5.2.3 Acute $O_3$ effects on leaf antioxidants

At this point only TAC and the  $\alpha$ -TOC analyses are finalised. TAC increases in broccoli leaves as a consequence of the acute  $O_3$  treatment ( $p=0,09$ ) (Fig 5.8 a), although a decrease in ASC content was observed in broccoli in 2007. The contribution of TOC to TAC is determined to be 2,2% for *Brassica oleracea* and 5,6% for *Brassica napus*, no significant differences were observed here. Further interpretation of these data by comparison with the acute  $O_3$  fumigation experiments of 2007 will only be possible when all antioxidant analyses have been completed.

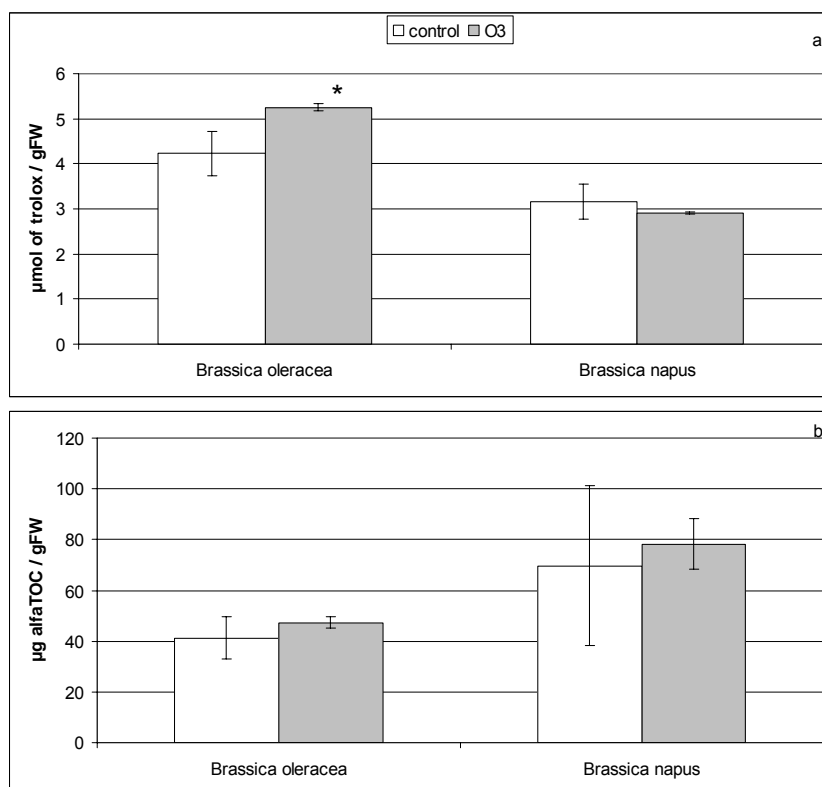


Fig. 5.8: Effect of a short, acute  $O_3$  exposure on (a) the total antioxidative capacity and (b) the  $\alpha$ -tocopherol concentration of oilseed rape and broccoli leaves

### 5.2.4 Acute O<sub>3</sub> effects on leaf glucosinolate content

From each chamber upper leaves of 10 plants/species were sampled for GSL analysis. The results for broccoli are shown in fig 5.9, for rapeseed leaves the results will be available for the meeting with the steering committee.

The short acute O<sub>3</sub> exposure caused a significant decrease of the total GSL content in broccoli leaves, mainly due to the reduction of the indol GSLs glucobrassicin and neo-glucobrassicin.

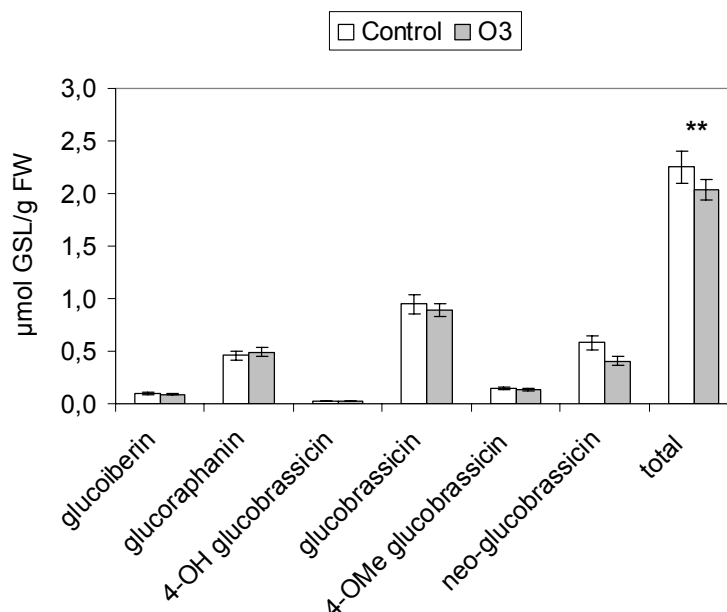


Fig. 5.9: Effect of a short acute O<sub>3</sub> exposure on the concentration of various glucosinolates (GSL) in leaves of *Brassica oleracea* cv Monaco

### 5.2.5 Molecular aspects

The protocol for RNA extraction was optimised and all samples have been extracted, converted into cDNA and pooled at this point. The use of primers designed for RT-PCR experiments with *Arabidopsis*-plants was examined. This was not successful at this point, hence only 2 housekeeping genes and 1 interesting test gene out of 8 were successfully amplified in combination with *Brassica oleracea* and *Brassica napus* cDNA-pools. CYP79 is an important gene in the biosynthesis pathway of some glucosinolates. But it wasn't significantly upregulated in either *Brassica oleracea* or *Brassica napus* (ratio's: 1,03 and 1,53). A new primer-design strategy based on the EST's of the microarray is being tested at the moment. After interpreting the microarray results of the OTC experiment (see further), a set of genes will be selected to examine the results of this acute exposure experiment more thoroughly.

## 5.3 Response of oilseed rape and broccoli to a long term, chronic increase in ozone concentrations (OTC) (2007&2008)

### 5.3.1 Climatic conditions

The average climatic data show little difference between OPs and OTCs (table 5.1), although the temporary differences could sometimes be quite significant. On the whole the weather conditions during spring and summer 2007 were not so

favourable for crop growth. Shortly after sowing oilseed rape, there was a very dry period that lasted almost 3 weeks and caused very irregular plant emergence on the experimental plots. Despite artificial watering, we obtained on average only 60 plants/m<sup>2</sup> in the OTCs and 90 plants/m<sup>2</sup> on the OPs instead of 120 plants/m<sup>2</sup>. However, in 2008 the climatic conditions have been very favourable, allowing a very homogenous emergence and growth of oilseed rape seedlings (Fig 5.10).

Table 5.1: Summary of the climatic conditions during growing season 2007 & 2008

Crops	Parameter	Units	2007		2008	
			OP	OTC	OP	OTC
Oilseed rape	Start - end	date	25/04 -21/08		21/04 – 08/08	
	Air temp	°C	16.5	16.6	16.2	16.5
	Soil temp	°C	16.6	16.3	15.9	15.6
	Vapour Pressure Deficit	kPa	0.45	0.41	0.54	0.47
	Global Radiation	MJ m <sup>-2</sup> d <sup>-1</sup>	13.7	11.8	13.91	14.25
Broccoli	Start - end	date	13/06 – 07/08		04/06 – 07/08	
	Air temp	°C	17.2	17.3	17.1	17.4
	Soil temp	°C	16.5	16.6	16.7	16.4
	Vapour Pressure Deficit	kPa	0.46	0.42	0.53	0.45
	Global Radiation	MJ m <sup>-2</sup> d <sup>-1</sup>	13.3	12.8	13.66	14.09



Fig. 5.10: Irregular emergence of oilseed rape in 2007 (left) compared to homogenous seedling growth in 2008 (right)

### 5.3.2 Ozone concentrations

In 2007 the ambient O<sub>3</sub> concentrations remained very low over the entire growing season (table 5.2) and no O<sub>3</sub> episodes have been recorded. This is also the reason why no visible O<sub>3</sub> injury was observed on either species, not even in the most elevated O<sub>3</sub> treatment. Nevertheless the critical O<sub>3</sub> level for yield reduction (AOT40 = 3 ppm h over 3 months for agricultural crops and 6 ppm h over 3.5 months for horticultural crops) was exceeded in the elevated O<sub>3</sub> treatments. In 2008 the ambient O<sub>3</sub> concentrations were clearly more elevated with several short O<sub>3</sub> episodes in July (fig 5.11), which increased the final AOT40 in the elevated O<sub>3</sub>

treatments by 25-50% in comparison to 2007. As such, over these two years already a wide range of O<sub>3</sub> exposure concentrations has been covered.

Table 5.2: Summary of the O<sub>3</sub> concentrations during oilseed rape and broccoli growth in 2007 & 2008.

Crops	Treatment	AOT40 (ppm h)		8hr mean (ppb)	
		2007	2008	2007	2008
Oilseed rape	OP	1.81	4.18	34	38
	NF	0.71	2.11	30	33
	NF <sup>+</sup>	5.66	10.74	46	53
	NF <sup>++</sup>	14.97	24.11	63	73
Broccoli	OP	0.76	1.86	32	34
	NF	0.38	0.74	29	29
	NF <sup>++</sup>	8.94	12.85	58	63

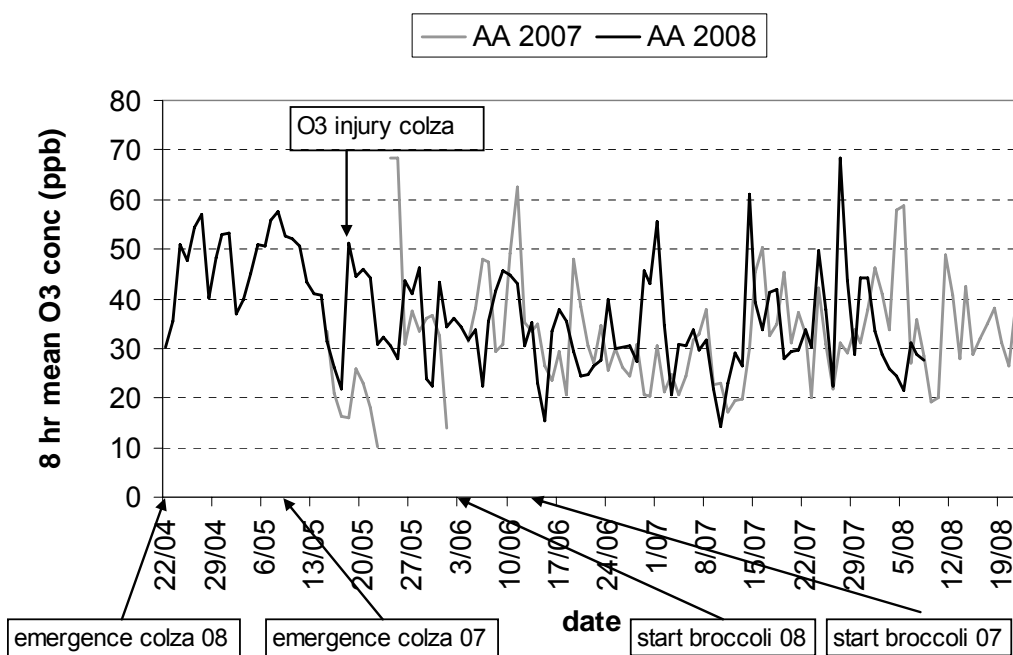


Fig. 5.11: 8 hr mean ambient O<sub>3</sub> concentrations (11 – 19 h local time) during growing season 2007 & 2008

Fig 5.12 shows the proportional increase in  $O_3$  concentrations in the fumigated OTC treatments compared to the unfiltered air. The slight decrease of the  $O_3$  concentrations in NF compared to the open field (AA) concentrations is due to breakdown processes in the ventilation-air duct system.

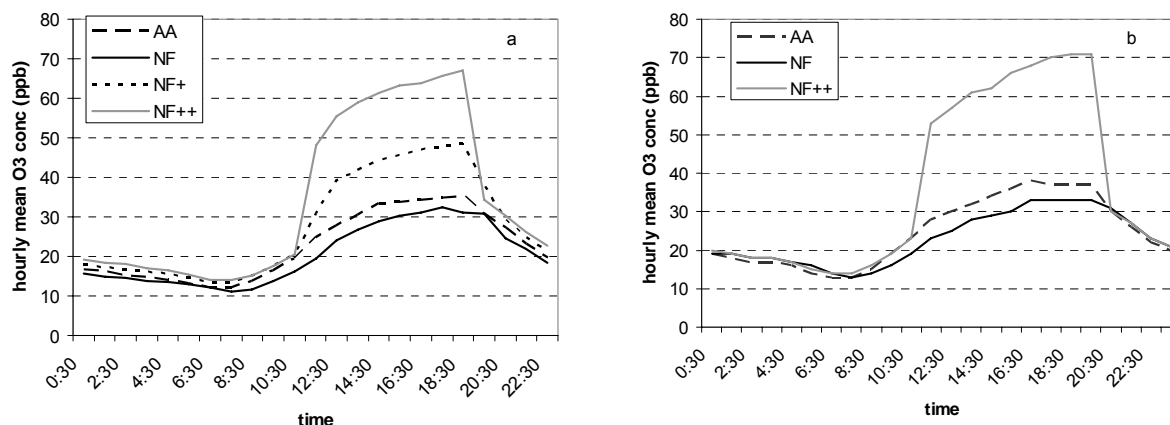


Fig. 5.12: Mean hourly  $O_3$  concentrations in ambient air (AA), unfiltered OTCs (NF) and elevated  $O_3$  OTC treatments (NF+ & NF++) over the growing season for (a) oilseed rape (2007) and (b) broccoli (2008).

### 5.3.3 Visible leaf injury

In 2007 no visible injury was observed in either treatment. The more elevated exposure level of 2008 did however induce leaf damage in the NF++ treatments. On May 21 the first  $O_3$  injury was observed on oilseed rape leaves in the NF++ treatment (fig 5.13) after a short  $O_3$  episode (compare to fig. 5.11). At final harvest intercostal chlorosis and stippling was observed on broccoli leaves of the NF++ treatment (Fig 5.14). These symptoms were not present in the NF treatments or on the OPs.



Fig. 5.13: First appearance of  $O_3$  injury on oilseed rape in 2008 (W21)



Fig. 5.14: O<sub>3</sub> injury on broccoli leaves observed in the NF<sup>++</sup> treatments at final harvest in 2008.

### 5.3.4 O<sub>3</sub> and chamber effects on crop development

In 2007 the leaf area index (LAI) was measured only once during vegetative and generative growth, whereas in 2008 these measurements were performed more frequently which gave a more comprehensive view on the evolution of the leaf area during the growing season (fig 5.15). Moreover, in 2008, at the start of flowering, an intermediate destructive harvest of the aboveground biomass of oilseed rape was performed consisting of 3 samples of 1m row length each, per chamber.

In 2007 the O<sub>3</sub> treatments did not induce any changes in leaf area of oilseed rape (fig 5.15 a) but in 2008 the more elevated concentrations clearly caused a reduction of LAI after flowering (Fig 5.15 c). This reduction of leaf area after maximum leaf area was achieved, seems to indicate an increased senescence rather than a reduction of initial biomass accumulation. For broccoli both in 2007 (fig 5.15 b, W27) and at several occasions halfway growing season 2008, LAI was reduced in NF<sup>++</sup> compared to NF (fig 5.15, d) but this effect seemed to have disappeared by the time of harvest

The chamber enclosure had a positive effect on LAI of oilseed rape (Fig 5.15 a). Probably the slightly more elevated air temperature in the OTCs stimulated the rate of leaf development in comparison to the field conditions. The opposite was true for broccoli (Fig 5.15 b,d).

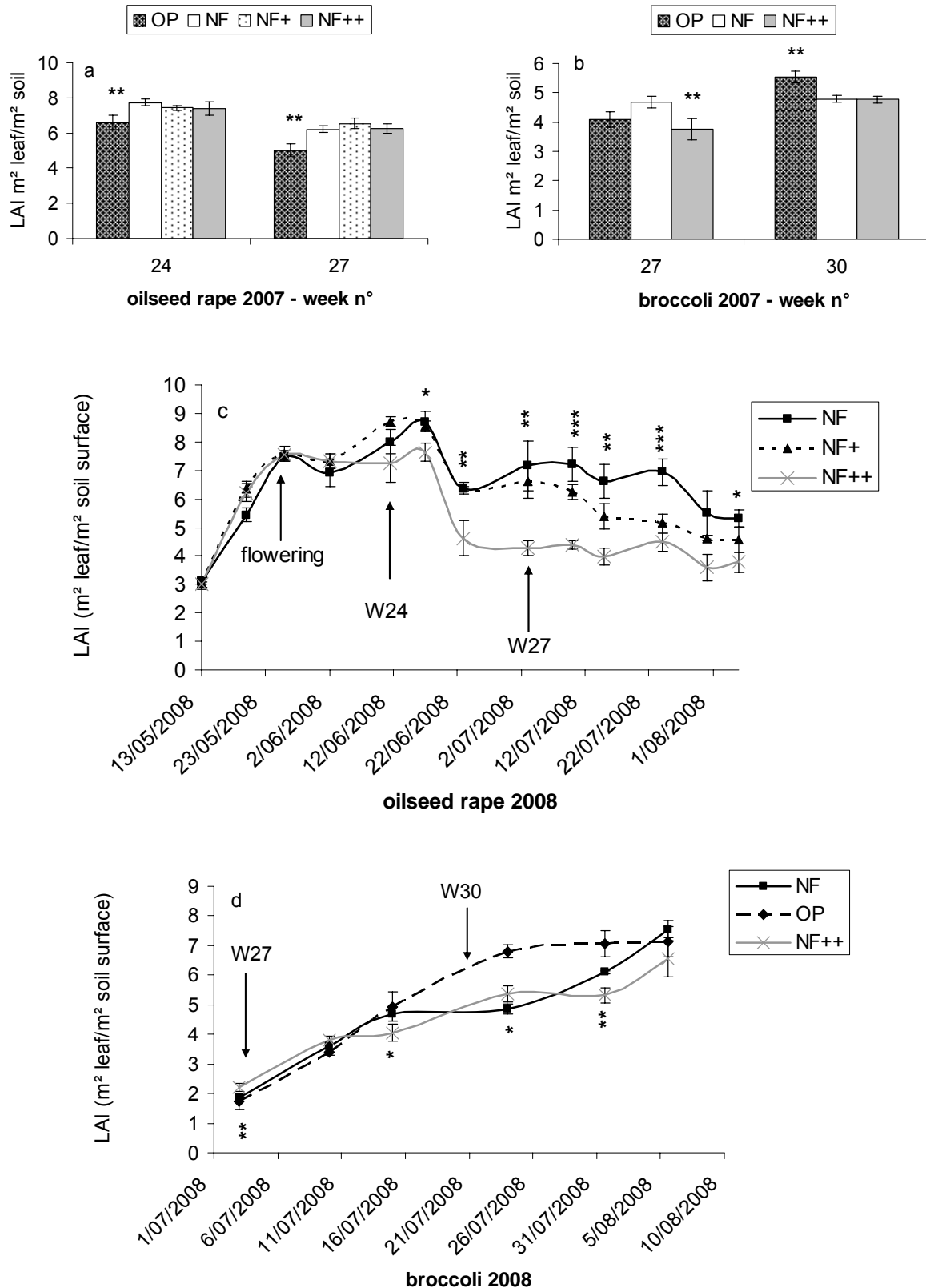


Fig. 5.15: Effect of elevated  $O_3$  exposure and chamber enclosure on Leaf Area Index (LAI) of oilseed rape (a, c) and broccoli (b,d) during their vegetative and generative growth in 2007 (a,b) and 2008 (c,d). For 2007 the significance of the difference with NF has been determined by two sample t test ( $* 0.05 < p \leq 0.1$ ). In 2008 significance of the  $O_3$  effect was determined by ANOVA.



The intermediate destructive assessment of the aboveground biomass in 2008 confirmed the LAI data of oilseed rape, though not significantly. Although this was not yet obvious from the non destructive LAI measurement at the time of flowering, at that moment the leaf and total DW already showed a tendency to decrease under the influence of elevated  $O_3$  (Fig 5.16). At the same time the % flowering increased: in NF<sup>++</sup> 25% of the plants were flowering, compared to 21 and 22% in the NF<sup>+</sup> and NF treatment.

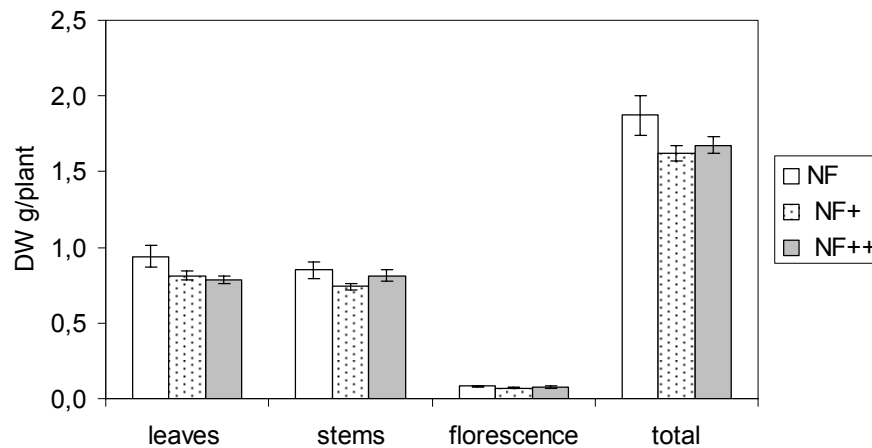


Fig. 5.16:  $O_3$  effect on aboveground biomass (g DW/plant) of oilseed rape at the onset of flowering (26/05/2008, W22). No significant  $O_3$  effects were determined ( $p > 0.10$ , GLM)

### 5.3.5 Long term $O_3$ effects on photosynthetic performance

#### *Brassica napus*

$CO_2$  assimilation at saturating light conditions on the youngest fully developed leaf confirmed in both years that, by the end of the growing season, the photosynthetic performance of these leaves was reduced by elevated  $O_3$  exposure (fig 5.17). The more frequent measurements in 2008 showed that this phenomenon was already evident at the end of flowering (W25) and the difference between treatments became more pronounced with the progress of senescence.



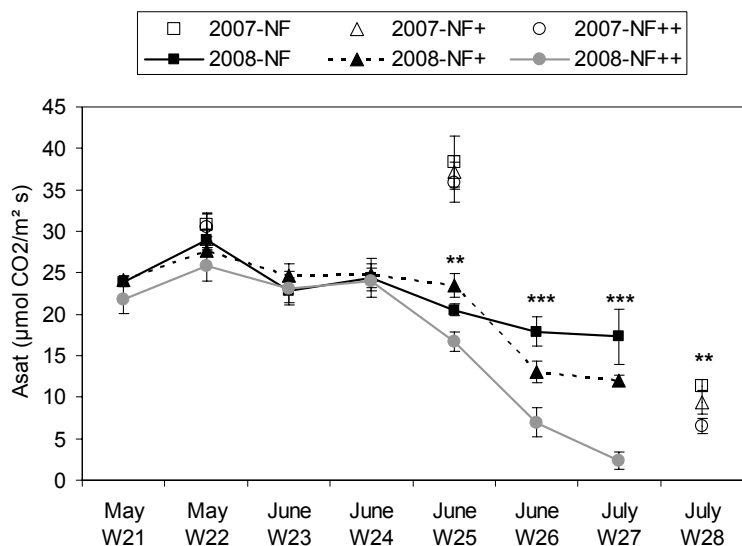


Fig. 5.17: O<sub>3</sub> effect on A<sub>sat</sub> of oilseed rape in 2007 and 2008. \*\* and \*\*\* indicate 95 and 99% sign O<sub>3</sub> effect (GLM). The pictures below give an idea of the developmental stages during the subsequent measurements in 2008.

The efficiency of photosystem II was assessed by chlorophyll fluorescence measurements both under saturating light ( $F'v/F'm$  = effective photochemical efficiency, Fig 5.18) and after dark adaptation ( $Fv/Fm$  = potential photochemical efficiency, Fig 5.19 a). To quantify the energy fluxes in the photosynthetic apparatus the PEA data were further analysed according to the theory of Strasser (for details see 3.2) (Fig 5.19).

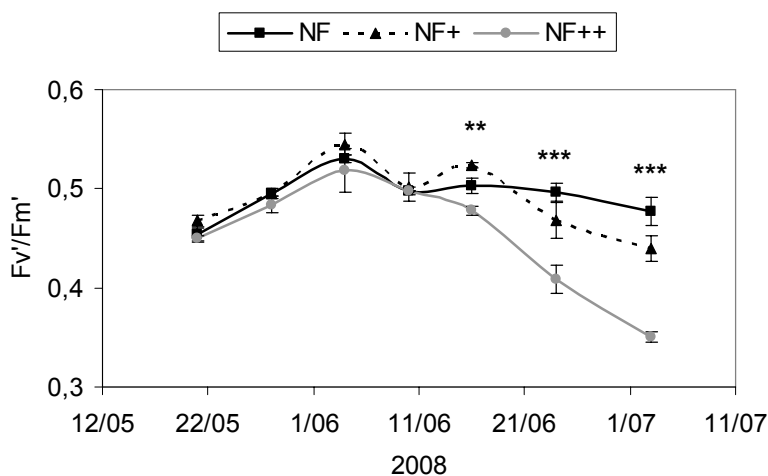


Fig. 5.18: Evolution of the effective photochemical efficiency of PSII of oilseed rape under saturating light conditions during 2008. \*\* and \*\*\* indicate 95 and 99% sign O<sub>3</sub> effect in 2008 (ANOVA)

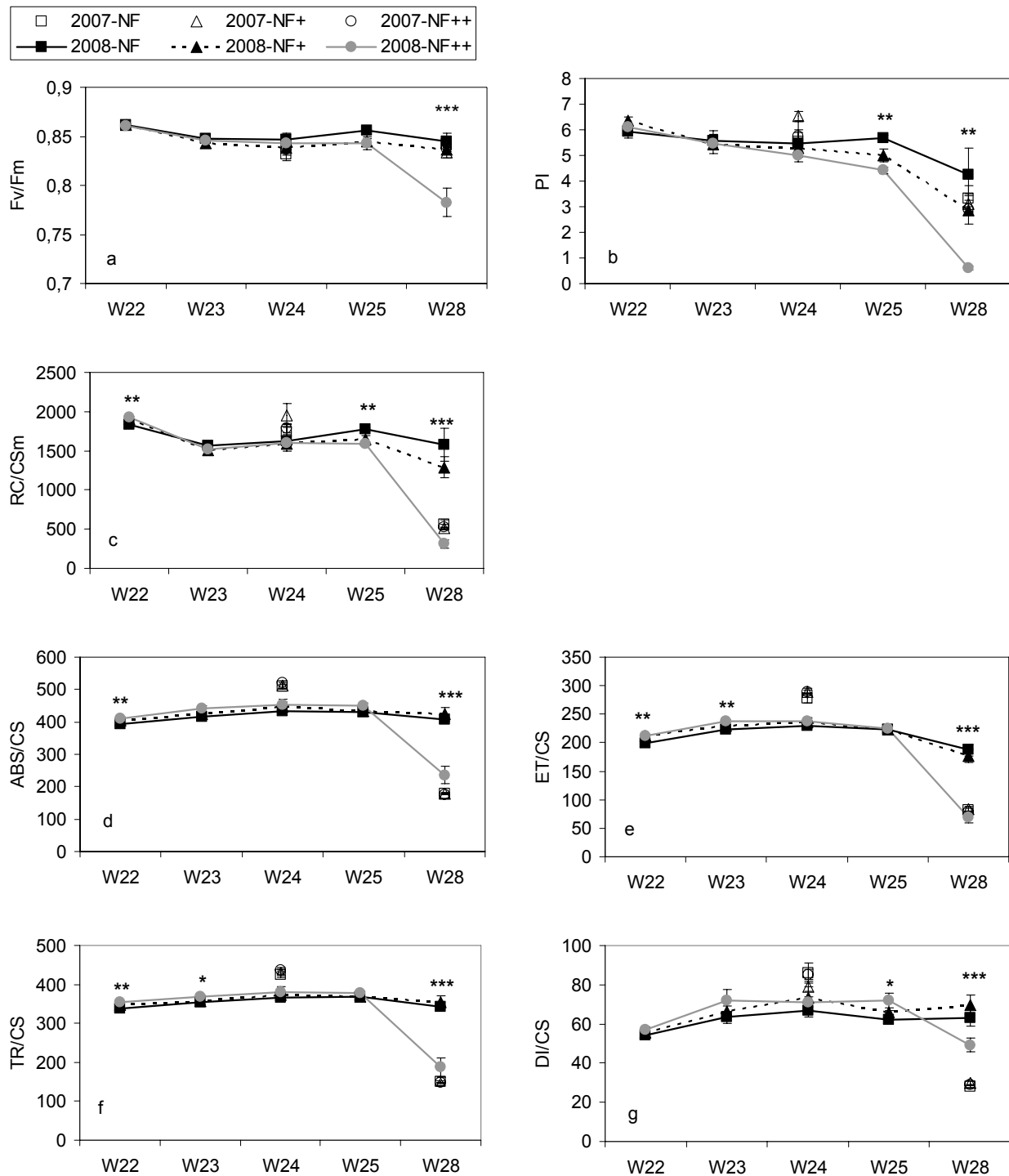


Fig. 5.19: O<sub>3</sub> effect on photochemical efficiency and energy fluxes of photosystem II as determined by chlorophyll fluorescence measurements: (a) potential photochemical efficiency Fv/Fm, (b) performance index PI, (c) photochemical reaction centres per leaf cross section RC/CS, (d) absorbed energy per leaf cross section ABS/CS, (e) electron transfer per leaf cross section, (f) trapped energy per leaf cross section, (g) dissipation energy per leaf cross section. \*\* and \*\*\* indicate 95 and 99% sign O<sub>3</sub> effect in 2008, there were no significant differences in 2007 ( $p > 0.10$ , GLM)

These chlorophyll fluorescence data confirm the decrease of photosynthetic activity after flowering (W25). This is related to a decrease of the photochemical reaction centres (chlorophyll) per leaf cross section (Fig 5.19 c). It is also remarkable that early in the growing season (W22) the increased  $O_3$  concentrations apparently induce the opposite effect. At that time RC/CS is highest in the NF<sup>++</sup> treatment and the energy fluxes in photosystem II are stimulated in comparison to leaves of the NF treatment (Fig 5.19 d,e,f). This may be an indication that oxidative stress initially causes a stimulation of metabolism to supply additional energy for repair mechanisms and defence responses.

In June 2008 several other photochemical parameters were assessed by fluorescence imaging performed by Prof R. Valcke. The photochemical efficiency of PSII under light conditions  $(F'_m - F_s)/F'_m$  e.g. does indicate an influence of  $O_3$ , but the images show a very large variation between subjects (Fig 5.20). Therefore in 2009 the measuring protocol will need to be adjusted. Furthermore, prof R.Valcke intends to improve the mathematical and statistical analysis of the images in collaboration with the department of Biostatistics of Hasselt University. Probably a PhD student will be assigned for this task.

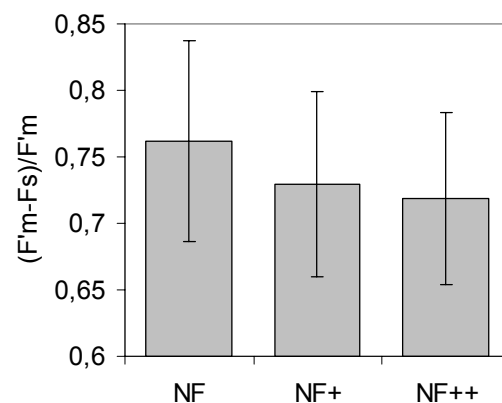


Fig. 5.20: Effect of  $O_3$  on the actual photochemical efficiency of oilseed rape under light conditions (2008).

The clear decrease in photosynthetic capacity and activity in 2008 coincides with a decrease in chlorophyll content (fig 5.21 b). It is obvious that for the older leaves this  $O_3$  effect appeared at an earlier stage during the growing season in comparison to the youngest upper leaves. In 2007 we measured no significant  $O_3$  effect on chlorophyll content of the upper leaves (Fig 5.21 a), but probably the measurements did not sufficiently cover the most crucial phase of senescence.

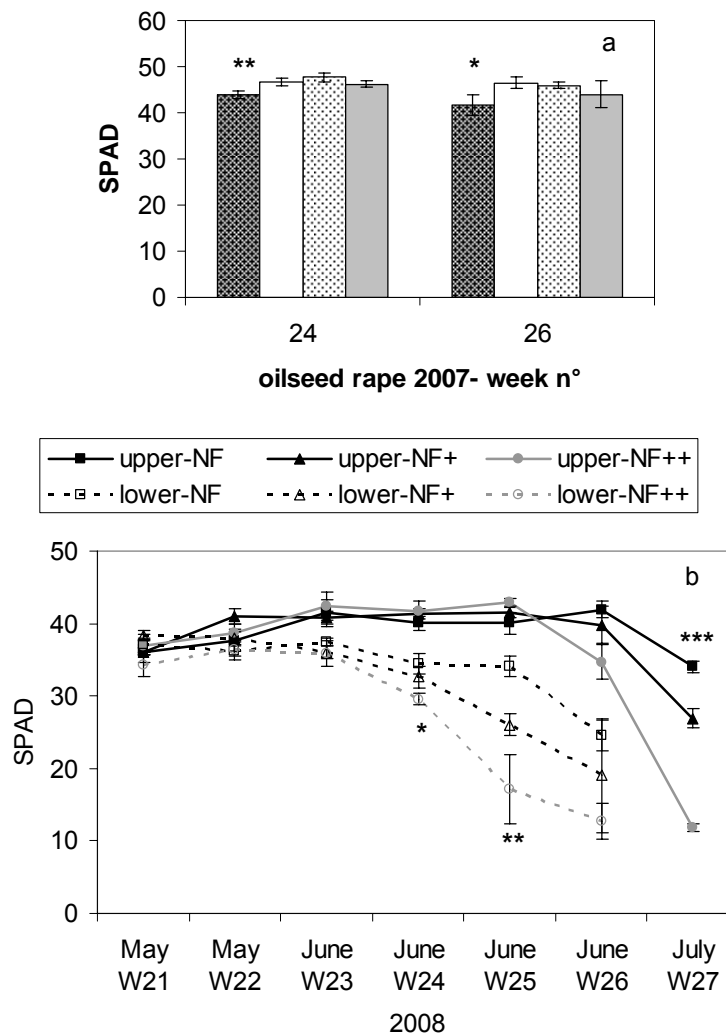


Fig. 5.21: Effect of elevated  $O_3$  exposure and chamber enclosure on chlorophyll conc. (SPAD) of (a) upper leaves of oilseed rape in 2007 and on both upper and lower canopy leaves in 2008. Significance of the difference with NF has been determined by two-sample  $t$  test (2007) or ANOVA (\*  $p \leq 0.1$ ; \*\*  $p \leq 0.05$ ; \*\*\*  $p \leq 0.01$ )

### *Brassica oleracea*

The data of 2008 show a perfect correlation between the effective photochemical efficiency and light saturated  $CO_2$  assimilation (fig 5.22 a,b).  $A_{sat}$  of the fully developed upper leaves of broccoli were not significantly affected by  $O_3$ , not even in 2008 (Fig 5.22 a). The same phenomenon was observed for  $F'v/F'm$  in 2008 (fig 5.22 b). It is clear that the  $O_3$  effect on photosynthesis is rather related to the long term effect on increased senescence since the only significant reduction could be measured on lower canopy leaves at the end of the growing season.

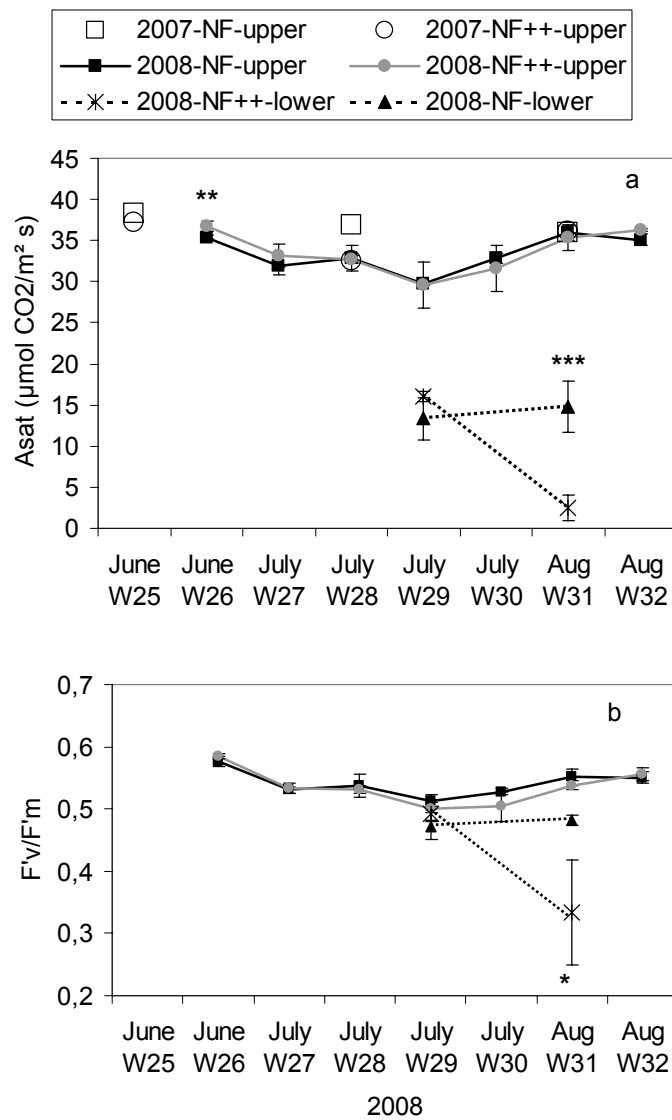


Fig. 5.22: (a) Light saturated CO<sub>2</sub> assimilation and (b) effective photochemical efficiency of broccoli leaves in 2007 and 2008 (\*, \*\*, \*\*\* indicates 90, 95 and 99 % dign O<sub>3</sub> effect, ANOVA)

In both years the potential photochemical efficiency (F<sub>v</sub>/F<sub>m</sub>), PI and the number of photochemical reaction centres per cross section of the upper leaves were clearly reduced under the influence of a season long exposure to more elevated O<sub>3</sub> concentrations (Fig 5.23 a,b,c). It appeared however that this did not reduce the absorbed photon flux and further electron transport (Fig 5.23 d,e,f), but there was a significant increase of dissipation energy (Fig 5.23 g).

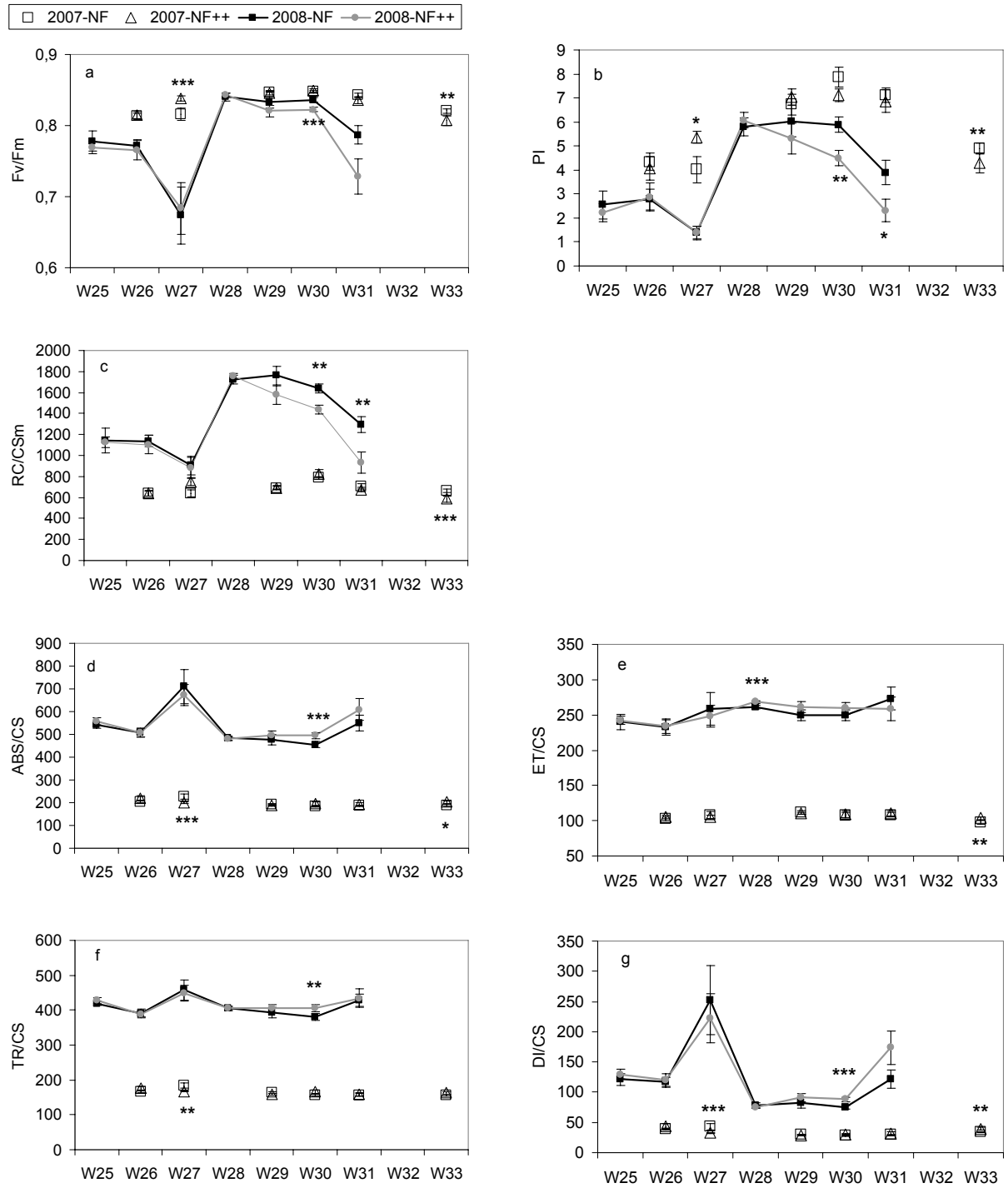


Fig. 5.23: O<sub>3</sub> effect on photochemical efficiency and energy fluxes of the upper leaves as determined by chlorophyll fluorescence measurements: (a) potential photochemical efficiency Fv/Fm, (b) performance index PI, (c) photochemical reaction centres per leaf cross section RC/CS, (d) absorbed energy per leaf cross section ABS/CS, (e) electron transfer per leaf cross section, (f) trapped energy per leaf cross section, (g) dissipation energy per leaf cross section. \*\* and \*\*\* indicate 95 and 99% sign O<sub>3</sub> effect in 2008, there were no significant differences in 2007 (p > 0.10, GLM)

The SPAD measurements on broccoli are only reliably related to the true chlorophyll content if the values remain below 55. Therefore only the last measurement of 2007 and those of 2008, that were all performed on older, lower canopy leaves are really worthwhile considering. They indicate a faster decrease of the leaf chlorophyll content in the NF<sup>++</sup> compared to the NF treatment (Fig a&b), both in 2007 and 2008.

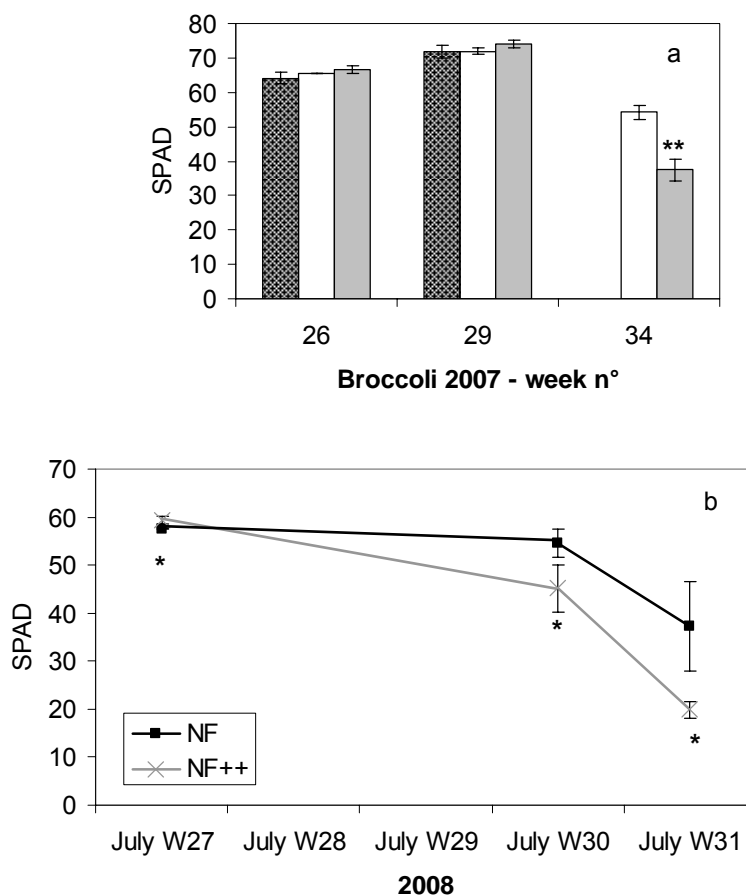


Fig. 5.24 Effect of elevated  $O_3$  exposure on chlorophyll conc. (SPAD) of (a) upper and lower (W34) leaves of broccoli in 2007 and (b) lower canopy leaves in 2008. Significance of the difference with NF has been determined by two-sample *t* test (\*  $p \leq 0.1$ ; \*\*  $p \leq 0.05$ ; \*\*\*  $p \leq 0.01$ )

### 5.3.6 Chronic $O_3$ effects on leaf antioxidant level

Both in 2007 and 2008 leaves of oilseed rape and broccoli have been sampled for analysis of ASC, GSH, TOC, TAC, GSL during the vegetative and generative growth. For 2007 most results are available, the major part of the samples of 2008 still need to be analysed. So the current interpretations are restricted to the data of 2007.

No significant  $O_3$  effects on ASC, GSH, TOC and TAC have been detected for oilseed rape at the first sampling date during vegetative development which is not surprising since the  $O_3$  treatment started less than 2 weeks earlier. For broccoli the first sampling occurred after 3 weeks  $O_3$  fumigation.

Total ASC of oilseed rape leaves shows a tendency to increase in response to  $O_3$  (fig 5.25 a -gen) whereas broccoli appears to respond oppositely (fig 5.25 b). The latter was also observed in the closed chamber experiment (fig 5.2b).

Total GSH and TOC levels of broccoli (fig 5.25 d,f,j) were considerably higher during generative development compared to younger leaves, but no significant O<sub>3</sub> effects were detected in either 2007 or 2008.

The total antioxidative capacity (TAC) of oilseed rape was increased in response to O<sub>3</sub> in the generative stage which was also apparent in broccoli, but before the appearance floescence (fig 5.25 g,h). For broccoli TAC was also higher in samples taken during generative growth compared to vegetative growth reflecting the same tendency as observed for all other antioxidants. For oilseed rape only one time point was investigated for TAC and therefore it could not be evaluated whether TAC levels showed a tendency to increase with age.



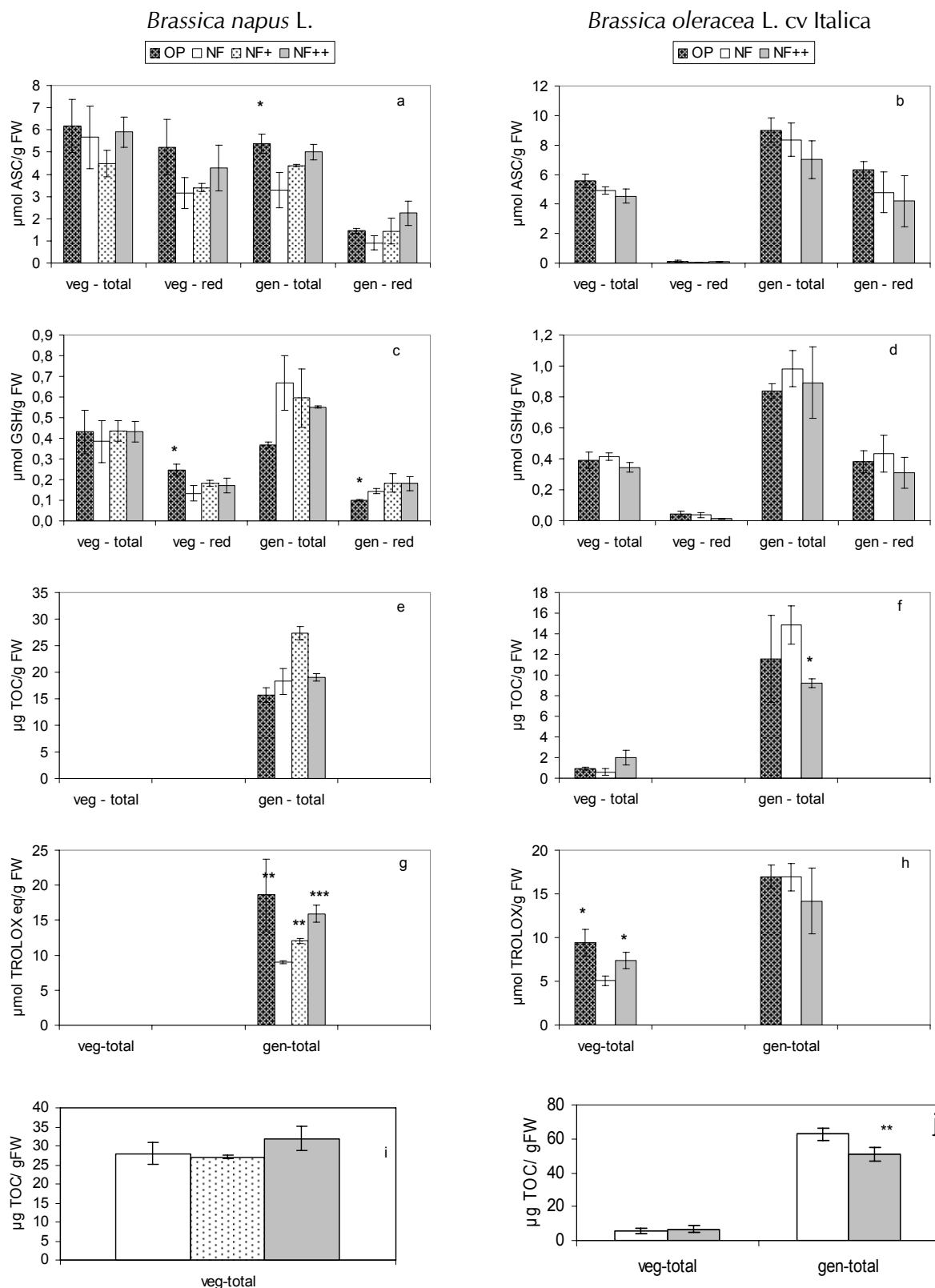


Fig. 5.25 Antioxidant concentrations in oilseed rape (a, c, e) and broccoli leaves (b, d, f) during vegetative (veg) and generative (gen) growth in 2007. For 2008 only the tocopherol concentrations are available (i,j). Total and reduced (red) ascorbate (ASC) (a,b) and glutathione (GSH) (c,d), total tocopherol (TOC) (e,f,i,j) and total antioxidative capacity (TAC)\* (g,h) have been determined. \*, \*\*, \*\*\* indicates 90, 95, 99% significant difference with NF (ANOVA, 2 sample t test & LSD)

### 5.3.7 Molecular O<sub>3</sub> effects at the leaf level

All RNA-extractions of both vegetative and generative leaves of broccoli and oilseed rape were completed on samples taken in 2008. RNA-extracts were pooled and used for microarray expression profiling currently conducted by Cogenics (USA). Upon retrieval of the results we will further analyse gene-expression using Real Time PCR, to establish O<sub>3</sub>-effects at a molecular level.

### 5.3.8 O<sub>3</sub> effects on yield quantity

#### *Brassica napus*

Harvest date of *Brassica napus* was determined by regular control of the moisture content of the seeds. Harvest is performed at a moisture content of approximately 10%. Seed yield for most spring canola cultivars is approximately 3 ton/ha. As such the yield on the OPs is comparable to real field conditions, (fig 5.26 a). In 2007 however the OTC yield was much less, which can be explained by the irregular plant emergence caused by serious drought immediately after sowing, resulting in only 60 plants/m<sup>2</sup> instead of 120. Moreover, the manipulation at harvest caused relatively more seed loss in the OTCs because these plants were already drier compared to the OPs. In 2008 the yield in the OTCs was much more comparable to the OPs thanks to the more favourable weather conditions and earlier harvest, after which the plants were further left to dry in the greenhouse.

Therefore the 2007 yield must be considered with some precaution, especially the NF treatment. In 2008 only the most elevated O<sub>3</sub> exposure caused a significant yield reduction of nearly 30% compared to the ambient and intermediate exposure level. This reduction (versus NF<sup>+</sup>) was comparable, though not significant, to 2007. The yield reduction was related to a reduction in 1000 seed weight (not significant, Fig 5.26 b) indicating that the individual seeds weighed less but their weight per 20 ml volume (density) was not affected (Fig 5.26 c). There was however a significant chamber effect on this parameter, maybe due to a difference in moisture content.

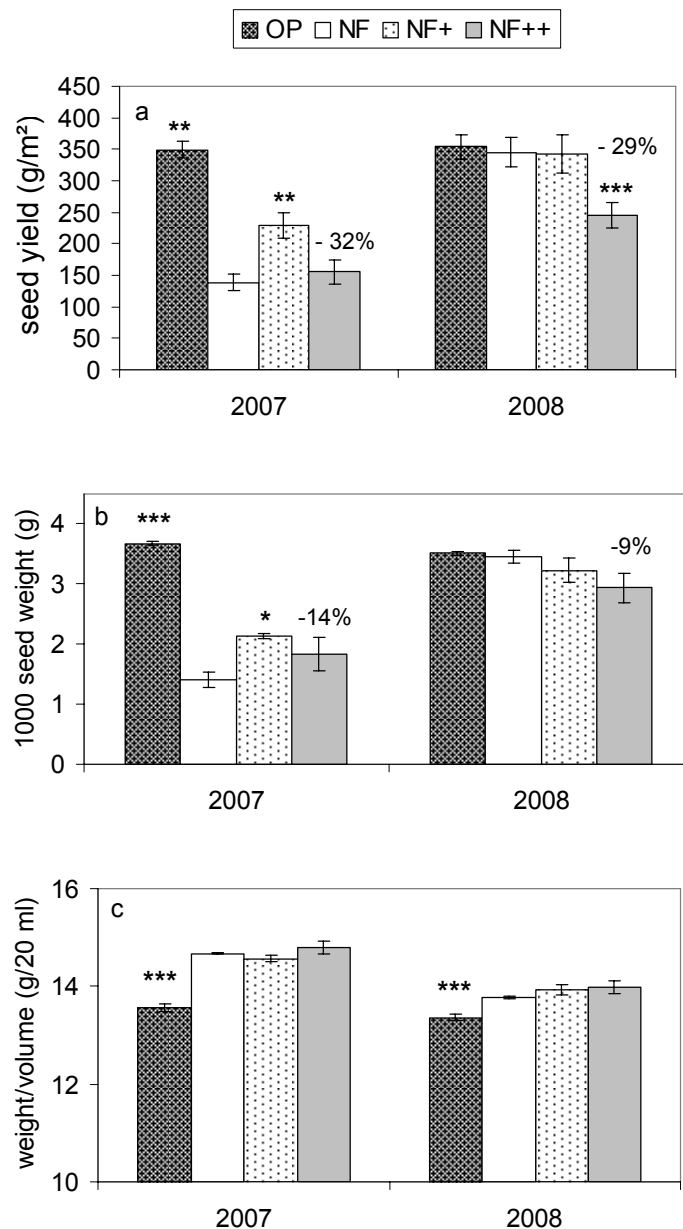


Fig. 5.26: Effects of elevated  $O_3$  and chamber enclosure on (a) seed yield, (b) 1000 seed weight and (c) seed density. Significance of the difference with the NF treatment was determined by GLM and Least Significant Difference (\*, \*\*, \*\*\* indicate 90, 95 and 99% significance level)

### ***Brassica oleracea***

At harvest, 12 plants/OTC or OP were cut at soil level. The broccoli vegetable was weighed to determine the fresh marketable yield. Leaves, stems and broccoli flower buds were further separated, weighed and dried at 75°C. Dry weight was determined and the samples were ground and stored for further analyses at VAR. Plants were harvested slightly earlier in 2008 compared to 2007.

Since broccoli was planted on the experimental plots after a homogeneous growth of the seedlings under optimal and controlled conditions, the variation within each population was much smaller in comparison to oilseed rape crops. Fresh marketable yield of broccoli vegetables was significantly reduced by elevated  $O_3$  in 2007, but

not in 2008 (Fig 5.27), despite the higher O<sub>3</sub> exposure level. This was also reflected in the dry weight of the individual plant parts and total aboveground plant weight (fig 5.28 a,b). The reason for this discrepancy is not clear yet. Perhaps the earlier harvest in 2008 had an influence, or the differences in O<sub>3</sub> uptake (flux) may provide an explanation.

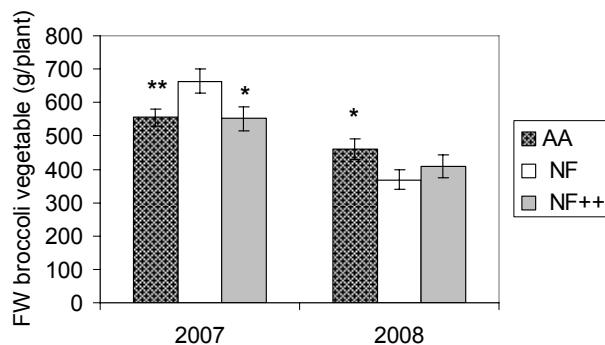


Fig. 5.27: Effect of elevated O<sub>3</sub> and chamber enclosure on fresh yield of broccoli vegetables. Significance of the difference with the NF treatment was determined by GLM (\*, \*\* indicate 90 and 95% significance level resp.)

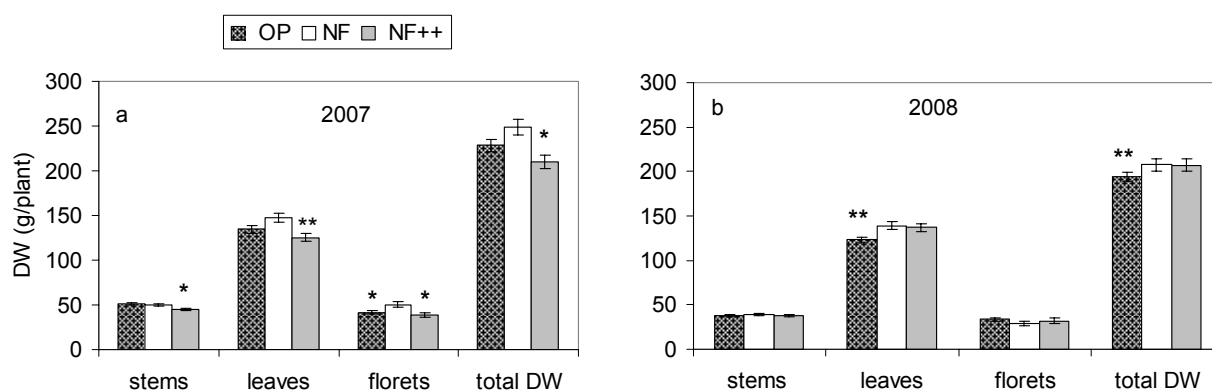


Fig. 5.28: Effects of elevated O<sub>3</sub> and chamber enclosure on dry weight of broccoli stems, leaves and vegetables an (a) 2007 and (b) 2008. Significance of the difference with the NF treatment was determined by GLM (\*, \*\* indicate 90 and 95% significance level resp.)

### 5.3.9 O<sub>3</sub> effects on yield quality

#### *Brassica napus*

Lipids and proteins are quantitatively the two most important fractions of oilseed rape seeds and account for more than 60% of the seed weight. The protein content of the seeds was significantly increased by the chamber enclosure, but there were no O<sub>3</sub> effects (Fig 5.29).

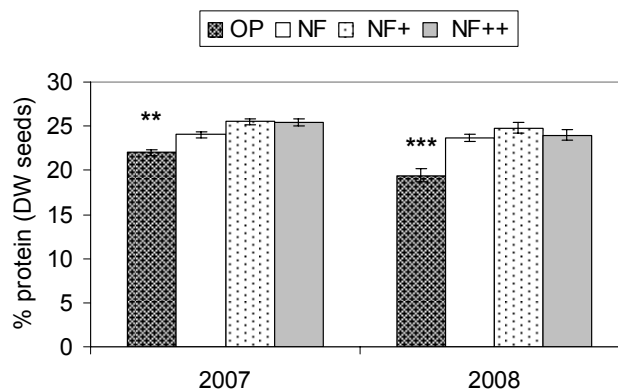


Fig. 5.29:  $O_3$  and chamber effect on protein content of seeds in 2007 & 2008. \*\* and \*\*\* indicate 95 and 99% significant difference with the NF treatment, ns is  $p > 0.10$  (ANOVA, LSD)

Next to the GSL content, the quality of rapeseed oil depends to a large extent on the fatty acid content. Oleic acid (C18:1) is by far the most present (approximately 60% of total fatty acids), followed by linoleic acid (C18:2) and linolenic acid (C18:3). These three fatty acids account for approximately 90% of total fatty acids. Chamber enclosure has an influence on the oil and fatty acid content, which may be related to the fact that the OP seeds were slightly less mature compared to the OTCs (Fig 5.30). With regard to the influence of elevated  $O_3$  exposure, it is again apparent that the NF results of 2007 do not follow the same tendency in comparison to 2008 (Fig 5.30 a,b). In 2008  $O_3$  induces clear decrease of oleic acid and of the oil %, accompanied with an increase in linoleic acid and % saturated fatty acids. The same tendency appears to be present for the NF<sup>++</sup> compared to NF<sup>+</sup> treatments in 2007, but NF is an outlier in this series. The increase of the linoleic acid content is important with regard to human nutrition because this is an omega 6 fatty acid that we cannot synthesize ourselves.

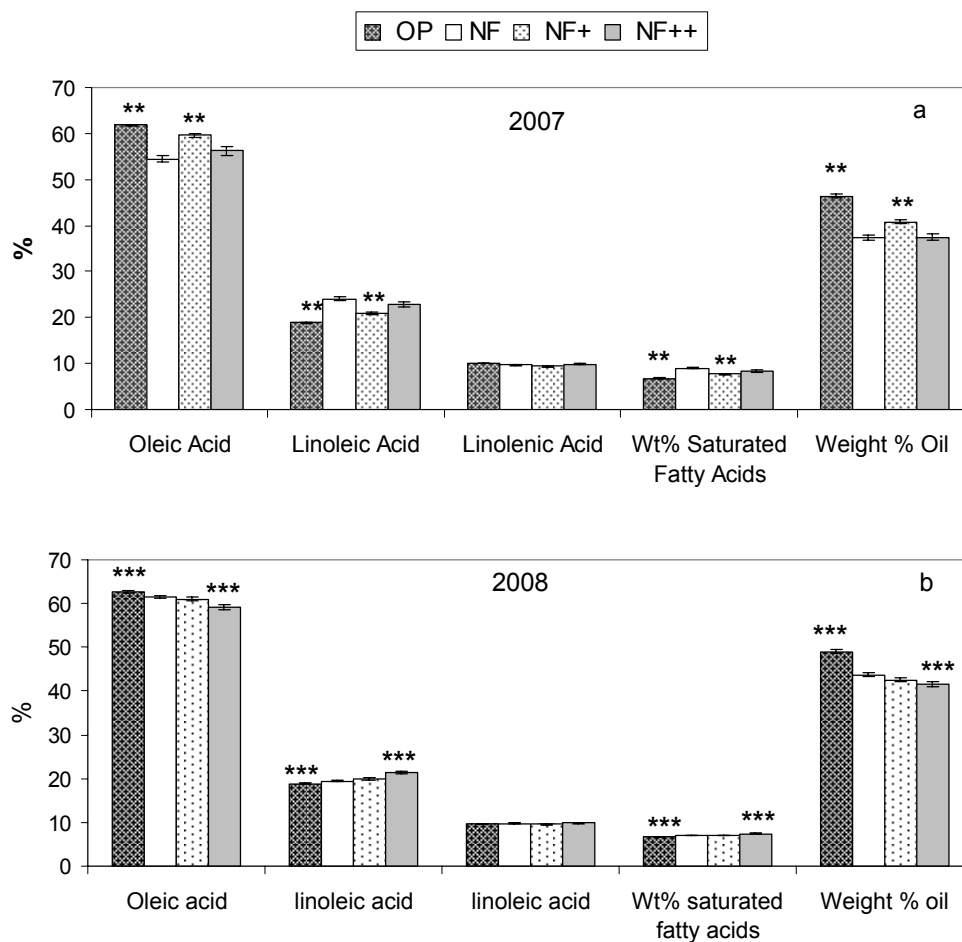


Fig. 5.30: : Effects of elevated  $O_3$  and chamber enclosure on fatty acid & oil content of oilseed rape in (a) 2007 and (b) 2008. Significance of the difference with the NF treatment was determined by ANOVA & LSD (\*, \*\*, \*\*\* indicate 90, 95, 99% significance level)

The seeds mainly contain progoitrin, gluconapin and 4OH-glucobrassicin.  $O_3$  did not cause a significant change in total glucosinolate concentration. Neither did we detect a significant shift in the relative GSL composition, comparable for both years (Fig 5.21).

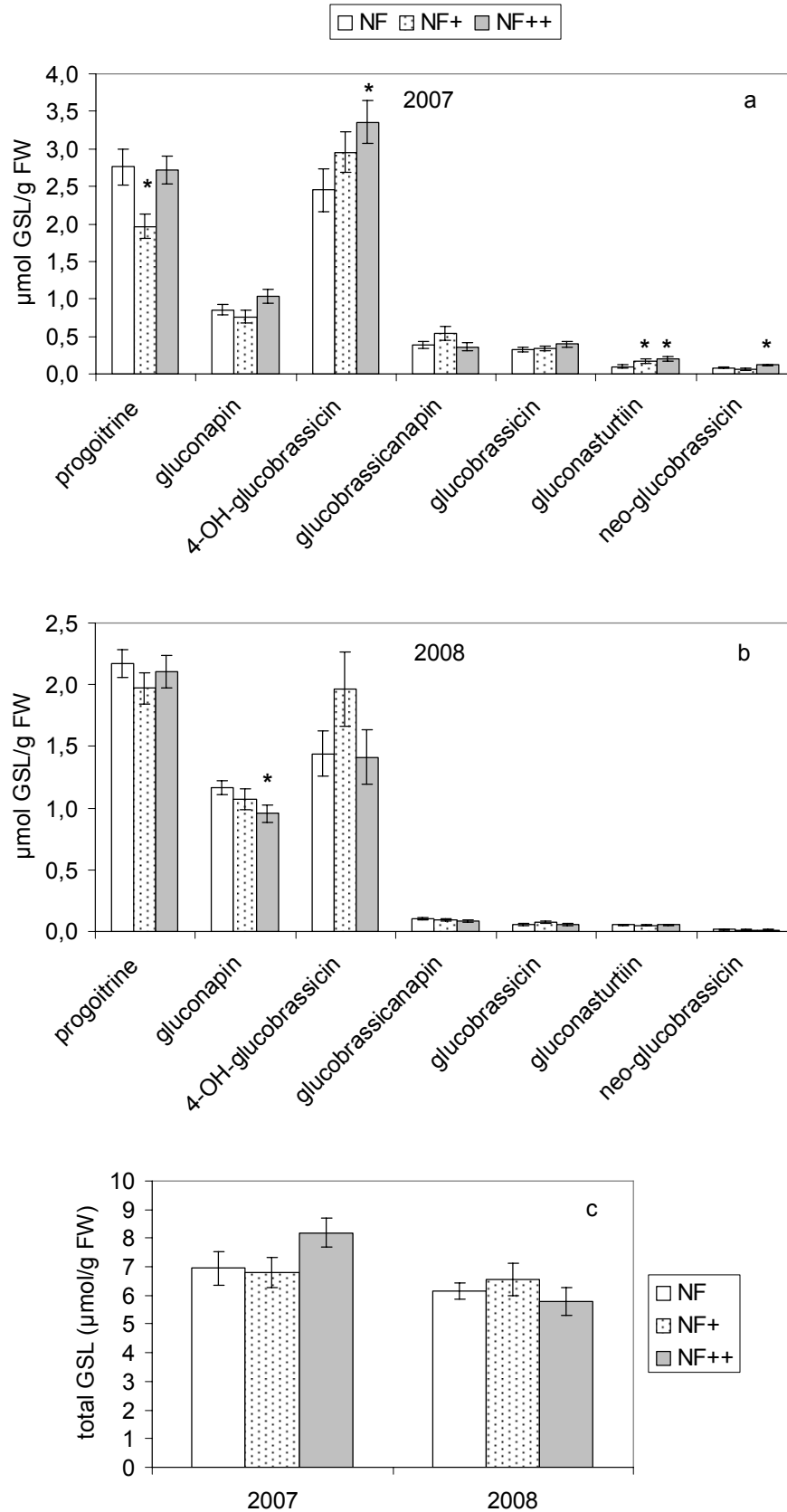


Fig. 5.31: O<sub>3</sub> effect on individual GSL content of air dried seeds in (a) 2007 and (b) 2008; the total GSL content (c) represents the sum if the identified GSLs. There was no overall significant O<sub>3</sub> effect on total GSL (GLM,  $p > 0.10$ ); LSD analysis on the individual GSLs did indicate some significant differences of the NF+ of NF++ treatment compared to NF (\*  $0.5 < p < 0.10$ )

### *Brassica oleracea*

As there were no significant interactions between the experimental year and the effect of O<sub>3</sub> (ANOVA  $p > 0.10$ ), data on the protein and GSL content have been pooled over both years. The protein content of broccoli plant parts was not significantly affected by an increase in O<sub>3</sub> exposure in 2007 and 2008 (Fig 5.32). Chamber enclosure did induce a significant increase of the stem and leaf N content at harvest. The highest protein content was found in the florets.

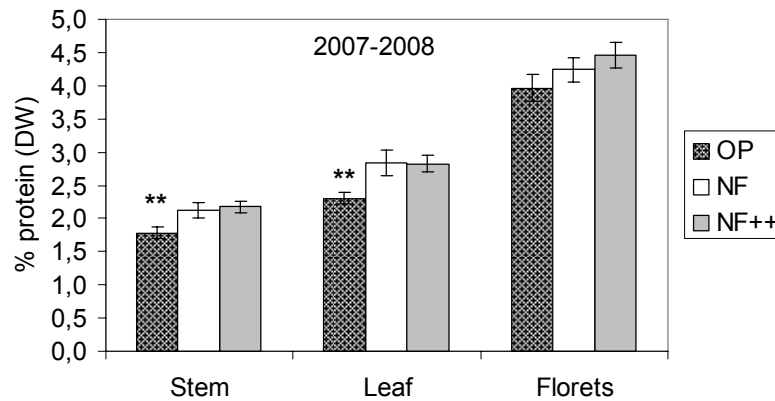


Fig. 5.32: Protein content of different broccoli parts, averaged over 2007 & 2008. \*\* indicates 95% sign difference with the NF treatment (GLM)

The long term increase in O<sub>3</sub> concentration caused a significant increase of the aliphatic GSLs glucoiberin and glucoraphanin, the latter being the most abundant GSL in broccoli vegetables (fig 5.33). This did not, however, result in a significant increase of the total GSL content as the effect seemed to be counteracted by a (not significant) decrease of the indole GSLs glucobrassicin and neo-glucobrassicin. This is an important shift since it is the ratio aliphatic/indol GSLs that is important with regard to human health. The anticarcinogenic activity of GSLs is mainly attributed to the aliphatic compounds such as glucoraphanin, whereas the health effect of indol GSLs is considered rather negative (carcinogenic). The aliphatic/indol ratio decreases as plants senesce.

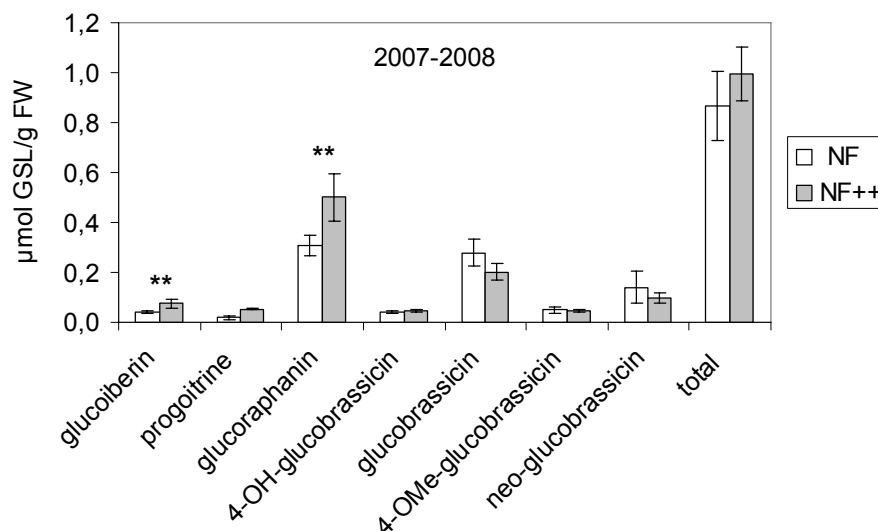


Fig. 5.33: Effects of elevated O<sub>3</sub> on glucosinolate composition and content of broccoli vegetables. Significance of O<sub>3</sub> effect was determined by ANOVA (\*\* is 95% sign)



In 2007 we did not detect any significant changes in vitamin C & E content of broccoli vegetables, neither as a consequence of the O<sub>3</sub> treatment nor due to the chamber effect (fig 5.34). The data for 2008 are not available yet.

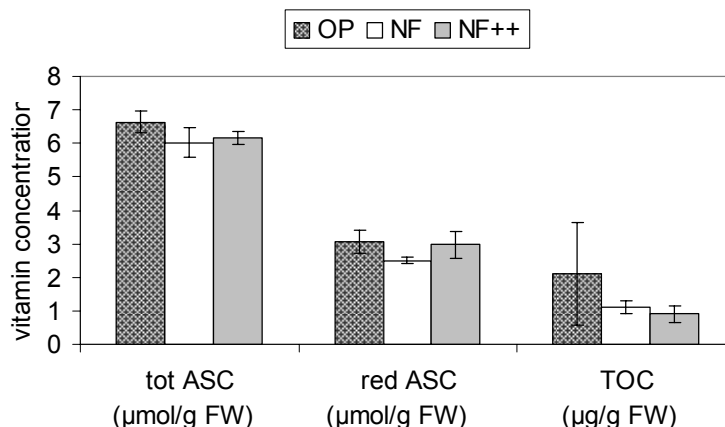


Fig. 5.34: Effects of elevated O<sub>3</sub> and chamber enclosure on the vitamin content of broccoli vegetables. There were no significant differences between the treatments (GLM,  $p > 0.10$ ).

### 5.3.10 Ozone flux modelling

The variation of plant specific stomatal conductance ( $g_s$ ) as a function of climatic conditions and phenology is essential for modelling O<sub>3</sub> uptake and to increase the reliability of the O<sub>3</sub> exposure-response relationship. Therefore measurements of  $g_s$  have been made over the entire growing season on the youngest fully mature leaves of oilseed rape and broccoli growing in full soil, both on the open-field plots and in the OTCs, under a wide range of climatic conditions.

The instantaneous stomatal O<sub>3</sub> flux ( $FO_3$ ) can be calculated based on the O<sub>3</sub> concentration and the simulated stomatal conductance for O<sub>3</sub>:

$$FO_3 = g_{stO_3} \cdot [O_3] \quad (\text{nmol m}^{-2} \text{ s}^{-1})$$

Stomatal conductance is simulated according to the Jarvis model as described in Emberson et al. (2000):

$$g_{stO_3} = g_{stO_3max} \cdot \max(f_{min}; f_{PAR} \cdot f_{temp} \cdot f_{VPD}) \quad (\text{mol m}^{-2} \text{ s}^{-1})$$

The stomatal model has been preliminary parameterised for oilseed rape and broccoli based on the LICOR and CIRAS measurements of 2007. However, these data cover only a limited range for VPD and temperature. So this parameterisation will need to be further refined when more data will be available. The following figures (fig 5.35) are an example of the simulated stomatal behaviour of oilseed rape in response to temperature, VPD and light intensity (PAR). These already indicate a large variation in O<sub>3</sub> uptake depending on climatic conditions.

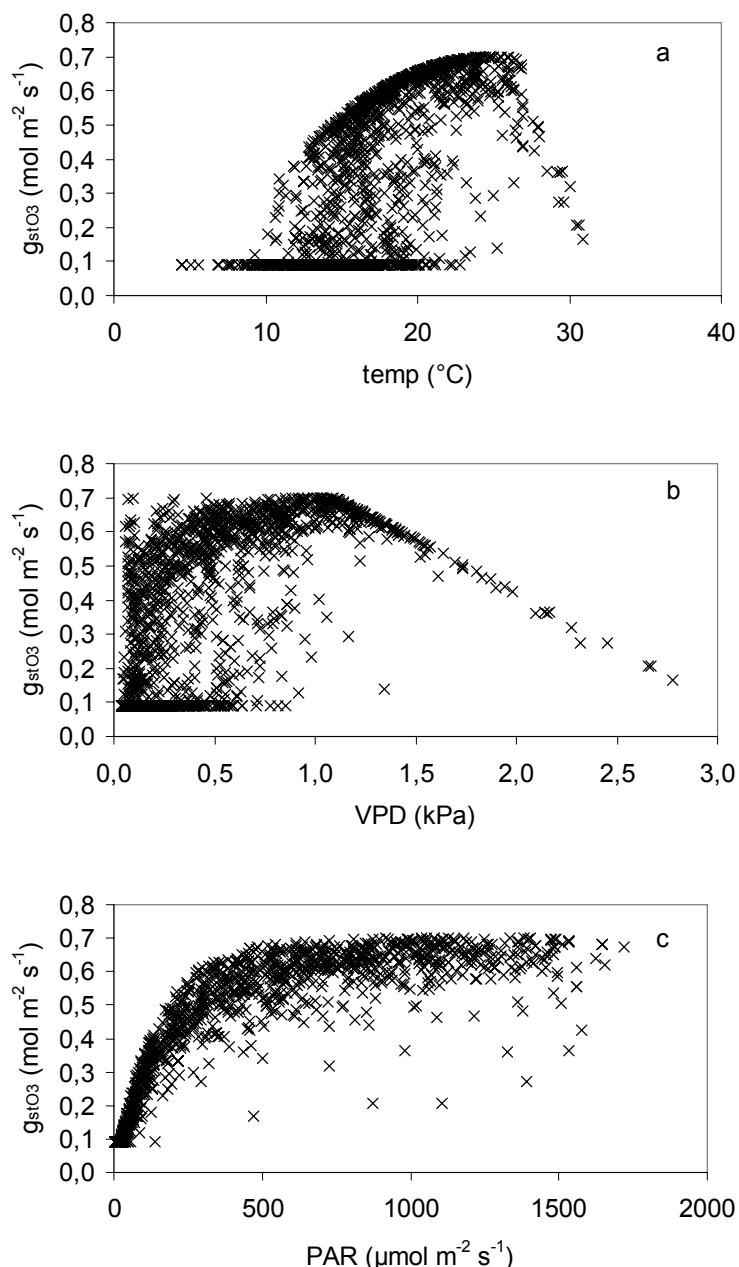


Fig. 5.35: Simulated stomatal conductance for O<sub>3</sub> ( $g_{stO_3}$ ) of oilseed rape in response to (a) ambient temperature, (b) vapour pressure deficit (VPD) and (c) photosynthetic active radiation (PAR).

It is assumed that there were no limitations in O<sub>3</sub> uptake due to soil water deficit as the experimental plots received underground irrigation when necessary. No correction has been made for any seasonal changes of the maximum stomatal conductance ( $g_{stO_3max}$ ).

Based on these assumptions and the provisional parameterisation, the cumulative O<sub>3</sub> flux (CUO) has been calculated as the sum of the instantaneous stomatal fluxes:

$$CUO = \sum (FO_3 \cdot 3600s) \cdot 10^{-6} \quad (\text{mmol m}^{-2})$$

As can be seen in fig 5.36 the calculated O<sub>3</sub> uptake is relatively higher for broccoli than for oilseed rape in comparison to the AOT40. An AOT40 of 8 ppm.h corresponded to nearly 75 mmol m<sup>-2</sup> accumulated O<sub>3</sub> uptake (fig 5.36 g) for broccoli compared to only 50 mmol m<sup>-2</sup> for oilseed rape (fig 5.36 f).

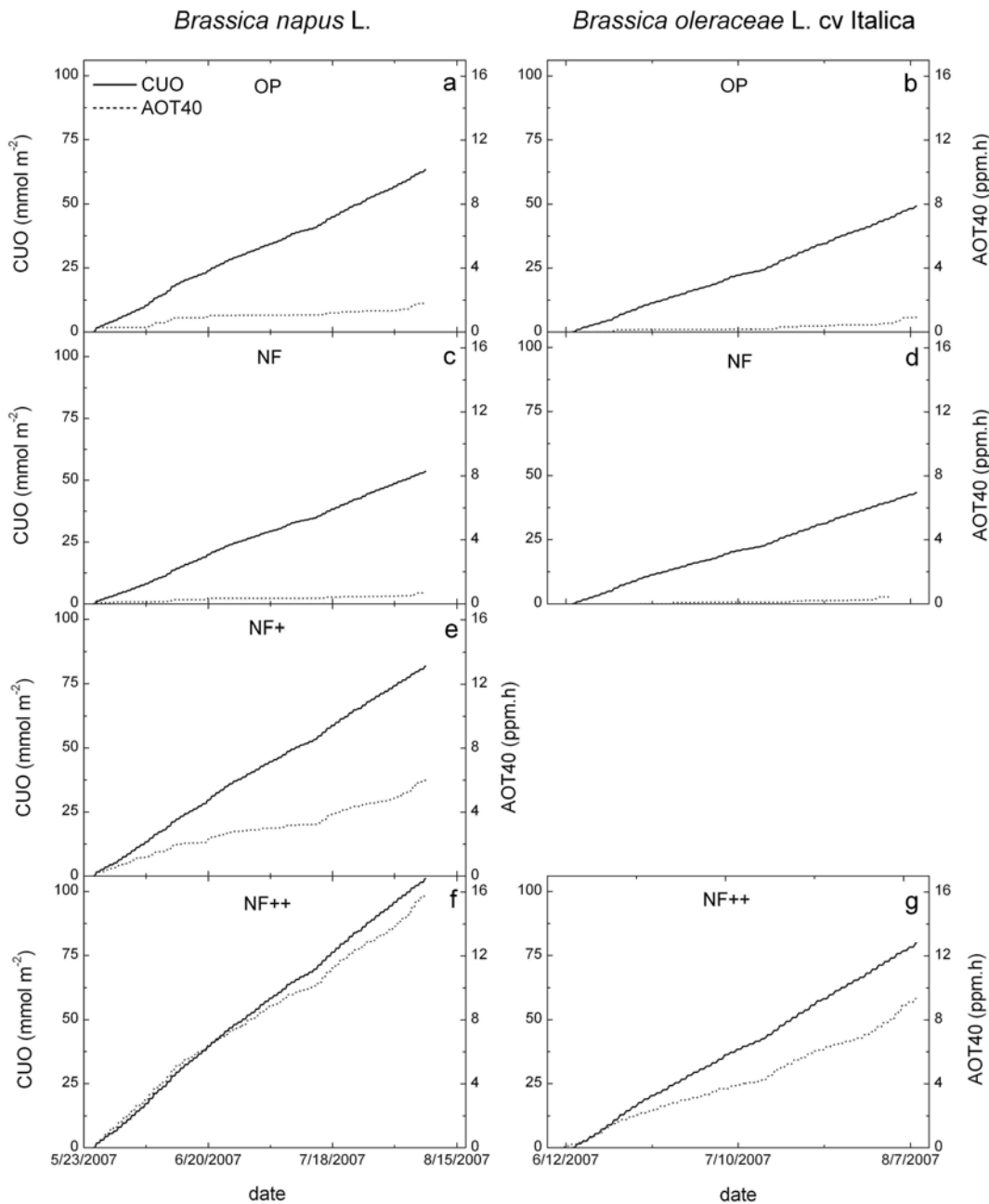


Fig. 5.36: Comparison of AOT40 and CUO for oilseed rape (a, c, e, f) and broccoli (b, d, g) in 2007

Table 5.3 represents the total accumulated O<sub>3</sub> uptake for broccoli and oilseed rape at final harvest. These data will be further explored to determine O<sub>3</sub> uptake-response relationships that can be used for O<sub>3</sub> risk assessments.

*Table 5.3: Cumulative O<sub>3</sub> uptake by oilseed rape and broccoli for the growing seasons 2007 & 2008*

Treatment	Oilseed rape		Broccoli	
	2007	2008	2007	2008
OP	63.27	76.68	49.16	58.56
NF	53.59	75.34	43.37	52.57
NF <sup>+</sup>	81.86	102.42		
NF <sup>++</sup>	105.01	132.85	79.91	94.55

## 6 Support to the decision

- The analyses of antioxidants and glucosinolates will provide data on the range of concentrations as they occur in vegetables and animal feed which is essential with regard to the evaluation of the “substantial equivalence” for risk assessment of genetically manipulated organisms. Moreover information on bioactive compounds in food are not always easy to find, although the need for these data is becoming increasingly important to researchers and the food industry as interest in potential health benefits expands, and as the consumers show more interest in diet and its potential impact on health and wellbeing. To this purpose our data may be submitted to the EuroFIR database, an EU funded Network of Excellence funded under the 6th framework Food Quality and Safety Programmes.
- Shift in levels of antioxidants and glucosinolates in vegetables and feedstuff due to O<sub>3</sub> pollution may have consequences for the food chain and the risks and/or benefits of such long term changes need to be evaluated. Until now air quality standards do not take these potential indirect effects into account.
- Data on the effects of O<sub>3</sub> on yield of oilseed rape and broccoli will provide information to improve air quality standards
- The ozone flux modelling is needed for further developing O<sub>3</sub> critical levels for the protection of yield and quality of agricultural and horticultural crops, as well as other vegetation. It has long been recognized that the flux-response relationship provides a more reliable estimation of the impact of tropospheric O<sub>3</sub> concentrations on plants which is very dependant on temporal and regional environmental circumstances. This work fits within the Convention on Long-Range Transboundary Air Pollution (LRTAP) and proceeds in close collaboration with the coordination centre of the International Cooperative Programme on Effects of Air Pollution on Natural Vegetation and Crops (UN-ECE ICP-Vegetation).
- The project aims to increase the knowledge of the plant-environment and plant-pathogen interactions which will surely contribute to developing new strategies to stabilise agricultural yield and quality in a fluctuating environment and changing climate.

## 7 Prospects and planning until the end of the project

During the first year of the second phase, the final OTC experiment will be performed, according to the same protocol as 2008.

Based on previous experience, some aspects may be reconsidered:

- Biochemical and physiological assessments during an O<sub>3</sub> episode have proven to be difficult to organise as they only last a few days. Destructive sampling is also rather limited since sufficient plant material needs to remain available for final harvest.
- Since several of the photosynthetic assessments confirm the same trends, the question is whether the number of applied techniques may perhaps be reduced
- For data analysis it is important to reinvestigate and pool the results of all 3 acute fumigation experiments.

To strengthen the scientific and policy related value of the project, following issues will be important:

- A major challenge will be to link the results of the molecular analyses to the observed biochemical and physiological changes.
- The O<sub>3</sub> responses will need to be linked to the flux modelling to provide reliable O<sub>3</sub> uptake-response relationships
- To estimate/model the effect of the qualitative changes of the consumable end products for the food and feed chain

Planning for 2009-2010:

Year 3	Month 1-3	continuation of data processing and analyses (C, P2, P3) field preparation for next growing season (C) second intermediate report on experimental year 2 (C, P2, P3) meeting of the follow-up committee (C) modelling stomatal ozone fluxes – data analyses (P3)
	Month 4-9	OTC ozone exposure of oilseed rape and broccoli + harvest - plant exposure in OTCs from sowing until harvest (C) - physiological assessments (C, P3) leaf sampling for molecular and biochemical analyses (C, P2)
	Month 10-12	analyses and data processing - antioxidant + TAC analysis (P2) - glucosinolate and quality analyses (C) - data processing of physiological assessments (C, P2) - data processing of environmental monitoring (C) modelling stomatal ozone fluxes (P3)
Year 4	Month 1-6	continuation of data processing and analytical analyses (C, P2, P3) finalising ozone flux modelling (P3) third intermediate report on experimental year 3 (C, P2, P3) meeting of the follow-up committee (C)
	Month 7-12	Final report (C, P2, P3) PhD thesis (C, P2, P3) Communication to SSD (C) Publications (C, P2, P3) Data transfer to EuroFIR and ICP-Vegetation (C) Dissimination of results (C)

## 8 Publications and presentations

- Vandermeiren K., De Bock M., Horemans N., Gielen B. (2008). Impact of tropospheric ozone on glucosinolate and vitamin C content of oilseed rape and broccoli. Proceedings of the 7<sup>th</sup> Workshop on Sulfur in Plants. Sirko A. (Ed). Warsaw, Poland. In press.
- Poster presented at the Workshop on Environment & Health 15&16/01/2007 – Brussels, Belgium  
"Impact of tropospheric 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 presentation at 7th Workshop on Sulfur Metabolism in Plants. 13-16 May 2008. Warsaw, Poland  
"Impact of tropospheric 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 22<sup>nd</sup> 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.