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Summary

With the growing importance of intensified agriculture to meet the demand for food and feed, the agricultural sector is increasingly relying on the use of pesticides. Together with other intensification practices, pesticides can assure the required increase in yield of agricultural crops as they prevent crops against weed competition on one hand and damage from pests and diseases on the other hand. Herbicides, which represent the largest proportion of all agrochemicals, contain active ingredients that inhibit plant-specific targets. However, it is unclear how crops cope with this chemical stress after exposure. Winter oilseed rape (*Brassica napus* L.) is cultivated for its oil-rich seeds, which are used in the production of vegetable oil for human consumption, press cake as fodder and the production of biodiesel. As it is most common in agricultural practice, winter oilseed rape is sown in early September, treated with the herbicide metazachlor one day after sowing and harvested in June. Metazachlor is a chloroacetamide herbicide, known to inhibit very long chain fatty acid synthesis in plants. This thesis aimed at revealing (i) the occurrence of herbicide-induced phytotoxicity in the non-target crop *Brassica napus* in the short-term, (ii) whether short-term phytotoxicity can affect the yield and quality of *Brassica napus* and its end products at the end of the growing season, and (iii) the role of cellular processes involved in xenobiotic detoxification and maintenance of the cellular redox state upon herbicide treatment. The effect of the application of metazachlor on *B. napus* cultivation was tested at three experimental scales: a mesocosm experiment (**Chapter 3**), microcosm experiments (**Chapters 4, 5 and 6**) and a field study (**Chapter 7**). A long-term mesocosm experiment revealed that metazachlor induced early phytotoxic effects at morphological level within one month after treatment (**Chapter 3**). As such, seedling survival was inhibited, growth was arrested and leaves displayed signs of disturbed development. Yet, at the end of the growing season, metazachlor did not induce significant changes in biomass production nor quality of the seeds, indicating the ability of rapeseed to recover from initial metazachlor-induced stress. To get more insight into the ability of crop plants to deal with the short-term herbicide-induced stress an analogous one-factorial microcosm experiment was conducted (**Chapter 4**). This study revealed that the initially impaired growth of *B. napus* within one month after application was

accompanied by cellular damage in the form of lipid peroxidation, which indicated the presence of metazachlor-induced oxidative stress. The activation of glutathione S-transferase and the decrease of internal metazachlor revealed that metazachlor was actively being metabolised by two-and four-weeks old *B. napus* plantlets. The simultaneous increased activities of enzymes involved in antioxidative defence enabled the crop to overcome the initial reduction of growth. The acute responses of *B. napus* towards the chemical stress were studied by the post-emergent application of metazachlor on 14-day-old plants (**Chapter 5**). The increase of potassium leakage revealed that the integrity of the cell membrane was impaired by direct interaction with metazachlor. Within 24 hours after treatment the increased activity of glutathione S-transferase and superoxide dismutase revealed the early activation of the detoxification metabolism and antioxidative defence machinery. However, these processes were disturbed by the induction of an oxidative burst 72 hours after treatment disturbed. Concurrently, plant growth stagnated, which was still visible seven weeks after treatment. This study revealed the dual role of hydrogen peroxide in signalling vs. damage. Monitoring photosynthesis and gas exchange in pre-emergently exposed plants at one month after application revealed that the efficiency of photosynthesis was safeguarded at metazachlor concentrations whereby pigment content was impaired (**Chapter 6**). Linking the effects observed at morphological and cellular level, the results derived from the short-term microcosm experiments indicate that the chemical energy derived from photosynthesis was consumed by processes involved in herbicide detoxification, maintenance of the cellular redox state and the protection of photosynthesis at the expense of growth. The long-term field study highlighted the necessity of pesticides use, not only with regard to reduced interspecies competition (*i.e.* weed competition), but also with regard to the prevention of intraspecific competition (*i.e*. between crops) (**Chapter 7**). Besides a minor decrease of quercetin-3-O-sophoroside and phosphorus in seeds derived from plants exposed to metazachlor, the vegetative and regenerative endpoint parameters were not influenced by metazachlor.

Samenvatting

Met het oog op voedselzekerheid, spelen pesticiden een steeds belangrijkere rol in de agricultuur. Samen met andere moderne landbouwpraktijken, levert het gebruik van pesticiden immers betrouwbare opbrengsten op doordat gewassen gevrijwaard worden van ongewenste onkruiden en ziektes. Herbiciden, die het grootste deel van de pesticiden afzetmarkt vertegenwoordigen, bestaan uit actieve bestanddelen die plant-specifieke processen verstoren. Maar het gebruik ervan gaat tegelijk ook gepaard met een blootstelling aan potentieel gevaarlijke stoffen en dus rijzen tegelijk ook vragen omtrent voedselveiligheid op. Tot op heden, is er weinig geweten over hoe gewassen met deze chemische stress omgaan. Winterkoolzaad (*Brassica napus* L.) wordt geteeld voor zijn oliehoudende zaden, die gebruikt worden in de productie van olie voor menselijke consumptie, perskoek als veevoeder en voor de productie van biodiesel. Eén dag na het inzaaien van winterkoolzaad, dat gebruikelijk in september gebeurd, wordt het veld besproeid met metazachloor. Metazachloor is een chloroacetamide herbicide dat de biosynthese van lange vetzuurketens in planten verstoort. De experimenten die in deze studie werden uitgevoerd beschrijven (i) het optreden van herbicide-geïnduceerde toxiciteit in het gewas winterkoolzaad vlak na behandeling, (ii) een mogelijke link tussen het voorkomen van toxiciteit op korte termijn en de opbrengst en de kwaliteit van winterkoolzaad en zijn eindproducten op lange termijn en (iii) de rol van cellulaire processen die betrokken zijn in de detoxificatie en in het behoud van de cellulaire redox status en de energiebalans, vlak na behandeling. Het effect van metazachloor op winterkoolzaad werd getest op drie verschillende schalen; aan de hand van een mesocosm experiment (**Hoofdstuk 3**), microcosm experimenten (**Hoofdstukken 4, 5 en 6**) en een veldstudie (**Hoofdstuk 7**). Het lange termijn mesocosm experiment toonde aan dat de stengel- en bladgroei, alsook de overlevingskans van het jonge gewas kleiner was één maand na toepassing van metazachloor (**Hoofdstuk 3**). Aan het einde van het groeiseizoen bleken de initiële toxische effecten van het herbicide echter geen effecten te hebben op de opbrengst van het gewas, noch op de kwaliteit van de zaden. Om meer inzicht te krijgen in de capaciteit van het gewas om te herstellen van deze initiële metazachloor-geïnduceerde toxiciteit, werd er een analoog, gecontroleerd experiment uitgevoerd in kleine microcosms

(**Hoofdstuk 4**). Uit deze studie is gebleken dat de vertraagde groei van winterkoolzaad, die werd waargenomen binnen de eerste maand na toepassing van het herbicide, gepaard ging met het optreden van cellulaire schade. Het voorkomen van lipidenperoxidatie van membranen in de bladcellen, duidde op de aanwezigheid van metazachloor-geïnduceerde oxidatieve stress. De activatie van glutathion S-transferasen en de afname van inwendige metazachloor, suggereerden dat metazachloor actief werd afgebroken door het twee tot vier weken oude gewas. De gelijktijdige toename van antioxidative enzymactiviteiten maakte het voor het jonge gewas mogelijk om de inwendige oxidatieve stress te beheersen. Deze capaciteit droeg bij tot het herstel van stengel- en bladgroei binnen negen weken na behandeling. De acute effecten van metazachloor werden bestudeerd door het herbicide toe te passen op twee weken oude planten (**Hoofdstuk 5**). De verhoogde concentratie van extracellulair kalium duidde op de rechtstreekse interactie van metazachloor met de celmembraan. Reeds 24 uren na behandeling werd er een verhoogde activiteit van glutathion S-transferase en superoxide dismutase waargenomen, die respectievelijk duidden op de activatie van het detoxificatie metabolisme en de antioxidative respons. Deze processen werden echter 72 uren na behandeling verstoord door een verhoogde concentratie aan waterstofperoxide. Op dit tijdstip werd er een afname van het gewicht waargenomen, dat zeven weken na behandeling nog steeds zichtbaar was. Deze studie benadrukte de tweezijdige rol van waterstofperoxide als signaleringsmolecule enerzijds en als schadelijke pro-oxidant anderzijds. Door monitoring van fotosynthese en gasuitwisseling processen in vier weken oude planten, bleek dat de efficiëntie van fotosynthese gevrijwaard bleef, zelfs onder de invloed van metazachloor concentraties die de pigmenten aantastten (**Hoofdstuk 6**). De effecten die werden waargenomen op morfologisch en cellulair niveau in de korte termijn microcosm experimenten duidden erop dat de chemische energie, afkomstig van fotosynthese, verbruikt werd door cellulaire mechanismen die betrokken zijn in herbicide detoxificatie, behoud van de cellulaire redoxbalans en de bescherming van het fotosynthese apparaat ten koste van de groei van winterkoolzaad. De resultaten uit de lange termijn veldstudie benadrukte het belang van pesticidengebruik, niet enkel in functie van het uitsluiten van competitie met onkruiden, maar ook in functie van het bepalen van de gewasdichtheid (**Hoofdstuk 7**). Naast een geringe afname

van quercetin-3-O-sophoroside en fosfor in zaden afkomstig van planten behandeld met metazachloor, werden de vegetatieve en regeneratieve eindpunt-parameters niet beïnvloed.

List of abbreviations

α	Photosynthetic rate in limited light conditions
A	Net CO_2 assimilation
A/ci	Intercellular to net CO_2 assimilation
AO	Ascorbate oxidase
APx	Ascorbate peroxidase
AsA	Ascorbate (reduced)
Ca	Calcium
Car	Carotenoids
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
Chl	Chlorophyll
ci	Intercellular CO_2 concentration
Cu	Copper
DAB	3,3'-diaminobenzidine
DCNB	1,2-dichloro-4-nitrobenzene
DHA	Dehydroascorbate (oxidised)
DHAR	Dehydroascorbate reductase
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTT	Dithiotreitol
DW	Dry weight
E	Transpiration rate
Ek	Minimal saturating irradiance
ETR	Electron Transport rate
ETR_{max}	Maximal Electron Transport rate
F	Fluorescence intensity
F_0	Minimal fluorescence (dark-adapted leaves)
Fe	Iron
Fm	Maximal fluorescence (dark-adapted leaves)
Fm'	Maximal fluorescence (light-adapted leaves)
FRAP	Ferric reducing antioxidant capacity
FW	Fresh weight
Fv/Fm	Maximal quantum yield of PSII
GPx	Guaiacol peroxidase
GR	Glutathione reductase
gs	Stomatal conductance

GSH	Glutathione (reduced)
GSSG	Glutathione disulphide (oxidised)
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
K	Potassium
MDA	malondialdahyde
MDHA	Monodehydroxyascorbate
MDHAR	Monodehydroxyascorbate reductase
Mg	Magnesium
Mn	Manganese
Na	Sodium
NBC	4-nitrobenzyl chloride
NBoC	nitrobenzoyl chloride
Npa	nitrophenylacetaat
NPQ	Fraction of non-photochemical quenching = $(F_m - F_m') / F_m$)
$O_2^{\bullet -}$	Superoxide radical
$^1O_2$	Singlet oxygen
$^{\bullet}OH$	Hydroxyl radical
P	Phosphorus
PAR	Photosynthetic active radiation
PrxR	Peroxiredoxin
qN	Non-photochemical quenching (NPQ of variable chlorophyll fluorescence)
qP	Photochemical quenching (fraction of open PSII reaction centres)
ROS	Reactive oxygen species
S	Sulphur
SOD	Superoxide dismutase
SPx	Syringaldazine peroxidase
TBA	Thiobarbituric acid
Y	Effective quantum yield of PSII
Zn	Zinc

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Chapter 1 Introduction

1.1. State of the art

1.1.1. Need for food and feed

Along with the estimated increase of the world population, the world is facing important issues regarding different aspects of globalisation, such as food security and environmental health issues. With a current world population of 7.3 billion, the United Nations expects the world population to increase by more than two billion people during the following 35 years, reaching 9.7 billion in 2050 and further increasing to 11.2 billion by 2100 (UN 2015). Together with the prospect of a growing world population, the need for food augments, thereby imposing great challenges for the agricultural sector to safeguard sufficient and healthy food supplies. Additionally, recent shifts in dietary patterns, the expansion of bioenergy crop cultivation and climatic disturbances impose even higher demands on global agricultural outputs. Overconsumption and shifts towards an increased portion of meat within the human diet, require more cereal supplies (Keyzer *et al.* 2005, Tilman and Clark 2014). Based on mass scale, 62% of the global crop production is used for human consumption, 35% for animal feed, indirectly and less efficiently consumed by humans, as meat and diary products, and 3% for bioenergy, seed and other industrial products (Foley *et al.* 2011). In addition to the predicted increase in population density, the world is facing challenges concerning climate change, in the form of shifts in temperature and rainfall patterns, leading to extreme and unpredictable droughts and flooding events and hence increased occurrence of natural hazards and disasters (IPPC 2014). The fifth Assessment Report of the Intergovernmental Panel on Climate Change has stated that global warming is already having a negative impact on agriculture, affecting major crops, livestock production and fisheries (IPPC 2014). With the agronomic sector being the most climate-sensitive economical sector (Muldowney 2013), the Food and Agriculture Organisation of the United Nations has estimated that over the previous ten years (2003-2013), climate change-associated economic losses were at least about 13 billion dollars for the crop sector and about 11 billion dollars for livestock (FAO 2015).

1.1.2. Food security and food quality: how to deal with future challenges?

Between 1972 and 1998, the demand for food has been doubled (World Bank, 2000). In the coming decades, the productivity of the agricultural sector has to increase even more with 60 – 100% by 2050 (FAO 2012) in order to meet the increasing demand for food and feed in the future. This can be achieved either by expanding the area of cultivation or by the intensification of agronomic practices on the existing arable land (Cassman 1999, Wirsenius *et al.* 2010). Agricultural intensification has dramatically increased in the previous decades. Ray and Foley (2013) have detected a shift in the world's arable land from a four-times faster increase in harvested land area in comparison with total standing cropland. Up to the new Millennium, the "Green Revolution" had encountered these challenges by the development and introduction of new agronomic technologies, such as the use of new crop varieties, augmented use of synthetic fertiliser, intensified irrigation practices, advanced tillage systems and pest control agents (Tilman 1999). Recently, investment shifted towards advanced biotechnology, which is promising in the development of either herbicide resistant crops or crop genes important in dealing with a changing environment.

1.2. Pesticides' benefits and constraints

1.2.1. Success of pesticides

Because of its broad set of advantages, the use of pesticides or agrochemicals has become an essential part of agriculture (Cooper and Dobson 2007). After all, agrochemicals prevent crop species from competition with weeds and damage from pests and diseases. The use of agrochemicals ensures a predictive yield. Yield losses of the major crops, such as rice, wheat, barley and maize, can run up to 70% if no weed, disease or pest control is practised (Oerke and Dehne 2004). With the use of agrochemicals, these losses can be prevented up to 40% (Cobb and Reade 2010). Next to the fact that pesticides prevent crop and livestock losses due to pests, they create the ability for farmers to grow at more particular times of the year. The application of pesticides is not labour intensive and easy to use on well-established and large-scale monocultures worldwide. The large assortment of products allows a broad range of pests to be battled and

offers the choice for enhanced specificity of pesticides, depending on the cultivated crop and the concerned pest. Moreover, the use of agrochemicals lowers the need for mechanical weed control, which in turn can have wider benefits as this results in a reduction of greenhouse gas emissions, decreased soil erosion and hence diminished water loss.

1.2.2. History and classification

Because of their synthetic origin, agrochemicals are described as xenobiotic compounds towards living organisms. Until the mid 1930's, crop protection compounds were mainly of natural or inorganic form. From then onwards, synthetic organic compounds were introduced that served as pesticides. The first chlorinated hydrocarbons, such as 2,4-D (2,4-dichlorophenoxyacetic acid) and MCPA (2-methyl-4-chlorophenoxyacetic acid), were developed between 1935 and 1950 and implemented as auxin-mimicking herbicides, inducing uncontrolled growth in susceptible plant species (Costa 1987). Due to their success, companies started to invest in research that led to the discovery of a wide range of chemical pesticides, which are currently available. Based on usage, herbicides represent the largest part of all pesticides, before insecticides, fungicides and others, with approximately one million tonnes applied in 2006 and 2007 (Grube *et al.* 2011). Herbicides contain active ingredients that can be defined by their ability to inhibit plant-specific targets such as enzymes or metabolites whereby unwanted weed species are not able to survive. The target site within a plant refers to the biochemical location at which an herbicidal compound potentially inhibits an important pathway and is assumed to be the most sensitive site to display a fast response. Such target sites include photosystem I and II, acetolactate synthase, glutamine synthase, tubulin assembly and organisation, cellulose biosynthesis and many more. Up to date, the commercial herbicides that are used in agriculture comprise about 20 biochemically different modes of action (Berg *et al.* 1999). An overview of all herbicidal active ingredients and their target sites is demonstrated in Figure 1.1, representing a close relation between compounds belonging to the same chemical family and their mode of action (Kaushik and Kaushik 2007).

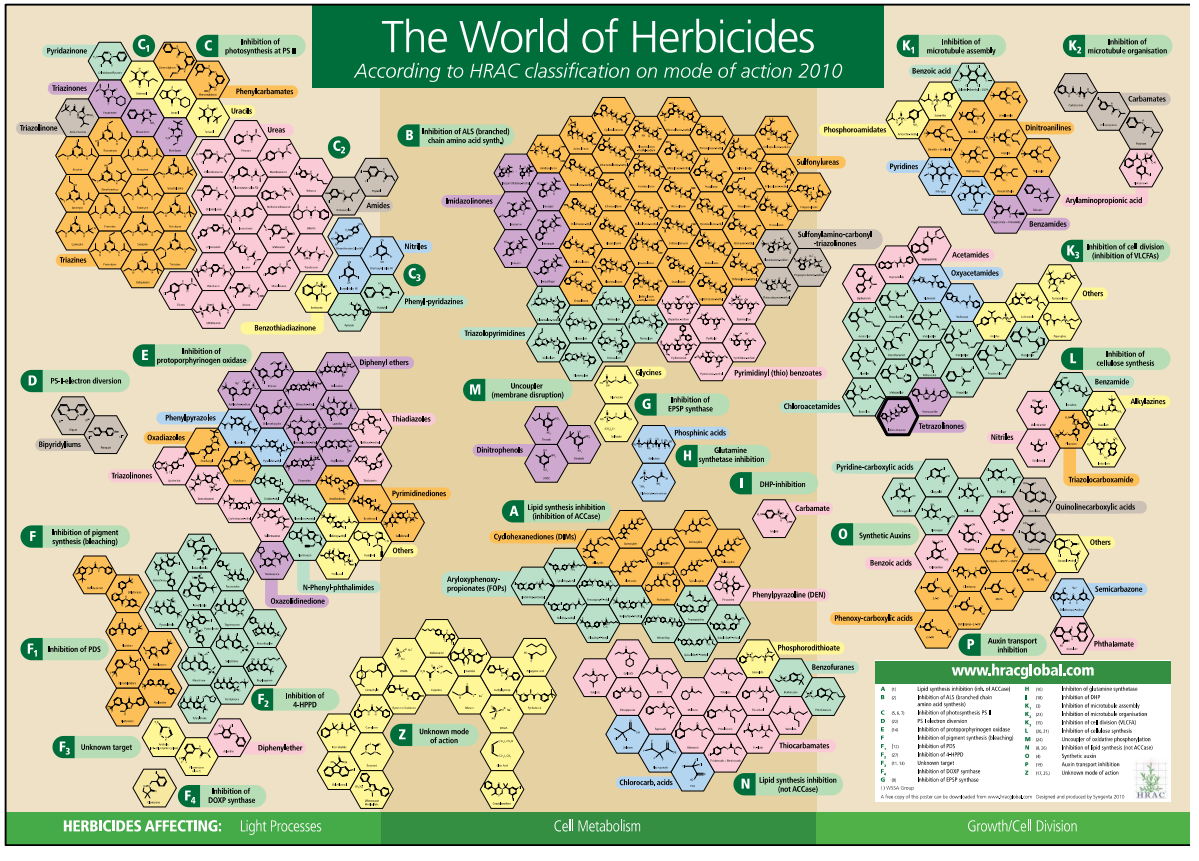

Figure 1.1. *An overview of all current active ingredients classified according to their mode of action and chemical resemblance. Metazachlor (accentuated in black) belongs to the family of the chloroacetamides.*

1.2.3. Discovery, development and regulations

Despite the many benefits of herbicide use in agriculture, weed resistance does occur (Preston 2004). This can be caused by modifications of the target site or by the increment in detoxification rates within the weed. Measures to prevent the occurrence of herbicide resistance include the use of herbicides with different target sites in mixtures or in rotational sequence. However, finding a new herbicidal target site could be promising. Up to date, it remains a scientific challenge to discover a compound with a new mode of action. The latest target site that has been discovered and commercially exploited is the enzyme 4-hydroxyphenylpuruvate dioxygenase (HPPD) and dates from 1991 (Rüegg *et al.* 2007). Applied chemical industries are continuously investigating new synthetic compounds for their potential use as pesticide. The search for new agrochemicals is based on chemistry and high throughput random screening in a stepwise selection process (Baker *et al.* 2002). Starting from large libraries of chemicals, which include compounds of either natural or pharmaceutical origin or even newly synthesised compounds, these compounds are randomly screened against weeds and crops in greenhouse experiments (Kudsk *et al.* 2003). Hereby, any growth regulatory or phytotoxic symptom is scored visually at regular intervals (Cobb and Reade 2010). The intention is to find compounds with promising biological activity towards weeds, which can be modified through conventional synthetic methods to advanced products. Subsequently, optimisation of compound synthesis and large-scale field trials are conducted. In the final step, product safety and registration of the compound is completed. The herbicides' mode of action is often determined after a chemical has passed all stadia of the selection and registration, which can take more than eight years (Cobb and Reade 2010). In Europe, the pesticide industry is obliged to follow procedures concerning safety examination of new pesticides for which they want to obtain authorisation (Verbeke *et al.* 2014). In this context, the European Commission (EC) approves the use of pesticides only if they have no harmful effects on consumers, farmers and bystanders; if the products do not cause unacceptable effects on the environment; and if the products are sufficiently effective against pests (European Commission 2009). In order to protect consumers from unacceptable high levels of pesticide residues in their food,

maximum residue levels have been set by the European Commission in Regulation NO 396/2005 and farmers are imposed to apply pesticides following the guidelines of good agricultural practice (GAP) (European Commission 2005).

1.2.4. Constraints accompanied by pesticide use

Up to date, human activities have led to the release of thousands of agrochemicals into the environment either on purpose or by chance (Bunzel *et al.* 2014a). The potential harmful effects of pesticides are partially caused by overuse or incorrect application due to inadequate knowledge about application method and doses. But most concerning is the unpredictable destination of pesticides and their residues into non-target species in the environment and subsequently into the food chain. First, these concerns are focussed on the effect on non-target crop plants, which are assumed to come into close contact with pesticides. Second, the destination of pesticides and their residues into the environment can be widespread depending on weather conditions and soil properties. Drainage and run-off enable pesticides and their residues to enter the ecosystem, thereby affecting the surrounding terrestrial and aquatic ecosystems, including non-target fauna and flora (Boutin *et al.* 2012). Eventually, the risk of pesticide residues entering the food chain via bioaccumulation arises. However, these risks only especially account for persistent substances.

1.2.4.1. *Agronomic and economic consequences*

Most studies in literature report pesticide-induced injury at early growth stages of the crop. As such, seed germination of soybean was impaired after treatment with fungicidal, insecticidal and herbicidal compounds (Aksoy and Deveci 2012). Crop injury from herbicides and their residues is influenced by several factors including herbicide persistence, crop rotation sequence and hence different herbicides used at a similar location, timing of application, tillage intensity, soil properties and weather conditions. These parameters can ultimately alter crop yield and quality of the crop and its end products (Felix *et al.* 2007). As such, excessive spraying of trifluralin, a germination-inhibiting herbicide, resulted in yield loss of winter oilseed rape at the end of the growing season (Majid *et al.* 2003). Herbicides inhibiting amino acid synthesis or photosystem II were reported to alter pigment profiles in crop plants (Alonge 2008, Carpenter and

Boutin 2010, Le Yin *et al.* 2008, Kummerová *et al.* 2008), leading to lipid peroxidation and a reduction in growth (Jiang *et al.* 2010). As such, pigment content in *Italian ryegrass* reduced within 30 days of exposure to glyphosate, its degradation product aminomethylphosphonic acid (AMPA) and the fungicide tebuconazole, but the efficiency of PS II remained unaffected (Serra *et al.* 2015). Reduced biomass production was also observed in cucumber shortly after exposure to 2,4-D, which resulted in a 19% reduction in yield at harvest (Gilreath *et al.* 2001). Nevertheless, recovery has been proven to occur in terms of biomass (Riemens *et al.* 2009) and metabolic processes (Follak and Hurle 2004) at the long-term. Whereas imazethapyr and acifluorfen induced visible injury and growth reduction of soybean in the short-term, a marginal reduction of yield was observed in the long-term.

1.2.4.2. *Ecological consequences*

The loss of natural habitats due to intensification of agriculture and urban expansion is putting increased emphasis on the importance of conserving field margins, hedgerows, woodlots, riparian habitats and wetlands. These habitats are important refuges for annual and perennial plant species and often provide food, shelter and movement corridors for other wildlife in agricultural landscapes (White and Boutin 2007). Pesticides originating from agricultural sources pose a significant threat to biodiversity and functioning of its surrounding terrestrial and aquatic ecosystems and ecosystem services as a result of mode of application, drainage or drift-off (Mitra and Raghu 1998, Stehle and Schulz 2015). Pesticides have been recorded to affect plant (Kleijn and Snoeijing 1997), invertebrate (Schäfer *et al.* 2012), bird and mammal communities (Grue *et al.* 1997). A Europe-wide study in eight countries comprising 13 components of agricultural intensification elucidated that the use of pesticides had the most consistent negative effects on biodiversity (Geiger *et al.* 2010). As such, the species diversity of local plants, ground beetles and ground-nesting farmland birds was reduced under the influence of insecticide and fungicide application. Model-based calculations on acute toxicity data of the crustacean *Daphnia magna*, a flagship species of the invertebrate community in the functioning of stream ecosystems, elucidated that the ecological risk to aquatic invertebrates from pesticide runoff was higher for crop cultivations of oilseed rape, potato or sugar beet than maize,

barley or wheat cultivations (Bunzel *et al.* 2015). Considering plant height and biomass, herbicide-induced sublethal phytotoxicity was observed in ten crops and ten non-target plant species in the short-term (Carpenter and Boutin 2010). Based on aboveground biomass in the long-term, recovery was recorded over time. However, for seven out of 12 species the reproductive endpoints were more sensitive to herbicide exposure than short- or long-term biomass endpoints. Therefore, the authors stated that reproductive endpoint parameters should be included in phytotoxicity testing.

1.2.4.3. *Consequences for human health*

As pesticides and their residues can enter the food chain either directly as a residual compound in crops or indirectly via entering the water cycle or via air pollution, they potentially impose a threat to human health. A recent study has indicated that pesticide residues are ranked among the top-three food safety issues of concern by a wide variety of stakeholders involved in the fresh food supply chain (Van Boxstael *et al.* 2013). Evidence about the link of pesticide exposure and the occurrence of human chronic diseases, including cancer, Parkinson, Alzheimer, multiple sclerosis, diabetes, aging, cardiovascular and chronic kidney disease augments (Firestone *et al.* 2005, Irigaray *et al.* 2007, Mostafalou and Abdollahi 2013). Pesticide screening in different *Brassica* vegetables resulted in the detection of 15 different pesticide residues, mainly insecticides, in 32% of the samples, of which 9% exceeded the maximum residue level (Łozowicka *et al.* 2012). Although the presence of these pesticide residues were not considered as a serious public health problem, the authors recommended continuous monitoring and a tighter pesticide regulation. Agricultural health studies including chloroacetamide herbicides found an association between cancer incidence and the application of alachlor and metolachlor (Alavanja *et al.* 2004, Lee *et al.* 2004). Oosterhuis *et al.* (2008) have described chloroacetamide-mediated inhibition of ABC transporters in human intestine cells, suggesting that these herbicidal compounds are able to modulate intestinal drug absorption.

1.3. An introduction to the herbicidal compound, metazachlor

1.3.1. Mode of action

Metazachlor is a chlorinated cyclic carbon structure that belongs to the chemical family of the chloroacetamide herbicides (Figures 1.1 and 1.2). Chloroacetamide herbicides are known to inhibit fatty acid biosynthesis, which can result in distortion of plasma membrane and cell wall synthesis (Fuerst 1987, Böger *et al.* 2000). Fatty acids, either saturated or unsaturated, constitute one of the many kinds of lipids present in plant cells and are mainly incorporated in membranes. Fatty acids are essential to plants, because of their structural and biochemical functions. Very long chain fatty acids are important components of the plant cell plasma membrane (plasma lemma) and are enriched in the leaf epicuticular waxes. For the latter, they are embedded in a matrix and ensure the hydrophobicity of the leaf structure. The cuticle is the main barrier against invasion of xenobiotic compounds and microorganisms and foresees the loss of water and solutes from the leaves. Disruption of fatty acid biosynthesis results in distortion of the plasma membrane possibly leading to alterations in permeability, transport and signalling functions.

Fatty acids are synthesised in both the chloroplast stroma and the cytoplasm. An overview of the different steps in fatty acid biosynthesis is represented in Figure 1.2. Acetyl coenzyme A is converted via malonyl-CoA into palmitic acid (16:0) with the help of two enzymes: acetyl-CoA carboxylase (ACC) and fatty acid synthase. The latter enzyme complex contains seven enzymes covalently bound to an acyl carrier protein, which transfers intermediates between the seven enzymes. Hence, seven cycles are needed for the condensation of seven additional C_2 units into one palmitic acid (16:0). There are two metabolic routes possible from palmitic acid. Either C_2 units are being added to palmitic acid in the cytoplasm by soluble condensing enzymes and endoplasmic reticulum-located elongases. This will yield very long-chained saturated fatty acids, which can be further processed into suberin and epicuticular waxes on plant surfaces. Palmitic acid can also be further desaturated by desaturases present in the chloroplast, yielding palmitoleic (16:1), oleic (18:1), linoleic (18:2) and α-linolenic acid (18:3).

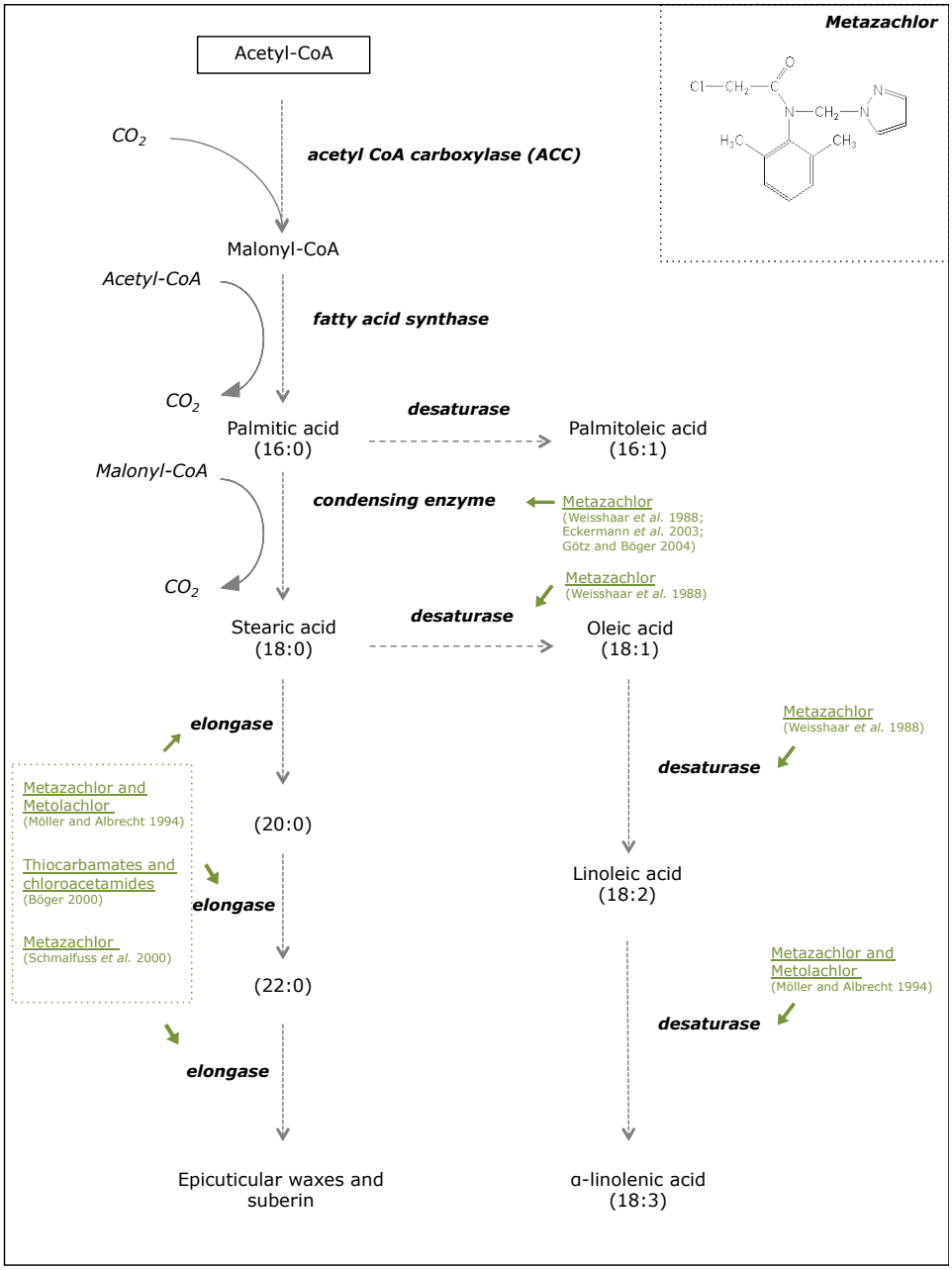

Figure 1.2. *Fatty acid biosynthesis in plants and possible inhibition sites for chloroacetamides as described in literature. Enzymes are represented in bold; the arrows indicate possible target sites of chloroacetamides, with the active ingredients underlined and the references represented between brackets. Scheme adopted from Cobb and Reade (2010).*

At least 70% of the total leaf fatty acids consist of the unsaturated α-linolenic acid (18:3), which itself makes up 40-80% of the lipid fraction in chloroplasts, thereby foreseeing fluidity to the thylakoid membrane.

During the eighties and nineties of the previous century much research has focussed on exploring the site of action of chloroacetamides, mostly by studying green alga *Scenedesmus acutus* (Weisshaar and Böger 1987; 1991, Couderchet and Böger 1993, Couderchet *et al.* 1998). The accumulation of palmitic acid (16:0) and the reduction of linoleic acid (18:2) under the influence of 5 µM metazachlor in *S. acutus*, suggested the inhibition of the carbon elongation step of palmitic acid (16:0) into stearic acid (18:0) and the desaturation steps into oleic acid (18:1) and linoleic acid (18:2) (Weisshaar *et al.* 1988). A decrease in oleic acid (18:1) was also observed in crop seedlings, such as maize and sorghum, exposed to 5 and 10 µM metolachlor, which coincided with the occurrence of growth injury in form of root and shoot growth inhibition (Wu *et al.* 2000). Inhibition of fatty acid synthesis has been observed in other crops, such as cucumber and barley as well (Fuerst 1991, Matthes *et al.* 1998). Möllers and Albrecht (1994) showed that chloroacetamides, such as metazachlor and metolachlor affected C18:2 desaturase and elongation from 20:1 in *Brassica napus* embryoids at concentrations of 0.01-0.025 mM. However, a review by Böger *et al.* (2000) stated that the formation of long chained fatty acids (up to C18) was not influenced by chloroacetamides, in contrast to the synthesis of very long chain fatty acids (VLCFA) (C20, C22, C24), which was affected. This correlated with cell growth inhibition. An in vitro elongase essay on microsomal preparation of leek seedlings suggested the inhibition of elongase steps in VLCFA synthesis and a tight binding between chloroacetamide metazachlor and the target enzyme (Schmalfuβ *et al.* 2000). Eckermann *et al.* (2003) suggested that the cysteine in the condensing enzyme is the primary common target of the chloroacetamide herbicides. Metazachlor binds irreversibly to the active site of a condensing enzyme involved in the first elongation step in VLCFA synthesis, and cannot be displaced by the substrate fatty acids (Götz and Böger 2004). In addition, the fatty acid substrates are easily removed from the condensing enzyme by metazachlor.

1.3.2. Mode of application and soil characteristics

Chloroacetamide herbicides were introduced in the early fifties and sixties, with allidochlor and propachlor as pioneers (Böger *et al.* 2000). Although some are replaced during history, this chemical family of herbicides is still widely used, with alachlor, propachlor, metolachlor and metazachlor as the most popular ones. In 2003 almost 14 million pounds of herbicides belonging to this family were used in the European Union member states, which accounted for 8% of total herbicide usage in the EU (Eurostat 2007). They are used on a broad spectrum of crops such as cabbage, tobacco, potato, maize, soybean, dry beans, rapeseed cultivations and many more (Fytoweb). Metazachlor is a chloroacetamide herbicide that is specifically applied on rapeseed (*Brassica napus, L.*) cultivation worldwide (European Commission 2012). Metazachlor can be applied in a pre-emergent and early post-emergent manner in crops in order to control grass and broadleaf weeds. In practice, it is recommended to apply maximal doses of 1.0 kg active ingredient per hectare (Fytoweb). In case of post-emergent application, chloroacetamides are absorbed by the seedling roots and transported to the upper parts of the plant. In case of alachlor, this transport occurs through the apoplast (Karavangeli *et al.* 2005).

Table 1.1. *Half-lives (days) for degradation of chloroacetamide herbicides in a sandy loam soil. Data based on Mozer et al. (1983).*

Temperature (C°)	25	25	25	25	15	5
Moisture (% w/w)	6	9	12	15	12	12
Propachlor	7.7	4.6	4.2	3.7	9.2	21.7
Alachlor	23.0	8.3	7.4	5.7	16.5	38.6
Metazachlor	24.2	18.1	13.2	11.6	29.2	77.0
Metolachlor	80.6	41.8	23.9	20.9	47.4	107.8

The half-live of metazachlor in organic soils ranges from 19 to 82 days (Mamy *et al.* 2008), resulting in a 25% presence in the upper 10 cm of the soil until four months after treatment (Rouchaud *et al.* 1992). However moist, temperature and the use of surfactants, such as oil or synthetic adjuvants, have an influence

on the retention time of metazachlor in the soil. As such, the retention time decreases with increasing moist levels and increases with lower temperatures and the use of surfactants (Table 1.1) (Mozer *et al.* 1983).

1.3.3. Potential harm of chloroacetamides in crops and surrounding ecosystem

Controlled laboratory experiments have demonstrated that chloroacetamides can induce crop injury. As such, root growth of 5- and 2-day-old pea and oat seedlings was reduced less than 48 h after exposure to 0.05 to 1.00 µM alachlor and metolachlor. Inhibited cell division was recorded in these seedlings and resulted in a reduced amount as well as a reduced size of root tip cells (Deal and Hess 1980). Reduced biomass production of barley was associated with reduced chlorophyll and carotenoid concentrations under the influence of 5- to 500 µM propachlor within 10 days after exposure (Kleugden 1980). Alachlor and metazachlor (50-100 µM) inhibited the growth of the green alga *S. acutus,* as a result of decreased photosynthesis, accompanied by membrane and thylakoid impairment (Weisshaar and Böger 1987).

In a long-term field trial, it was demonstrated that the degradation time of metazachlor in the soil ranged from one to three months (Kucharski and Sadowski 2011). The addition of oil and surfactant adjuvants reduced the degradation time in the soil and increased the level of residue in the soil and resulted in the presence of metazachlor in harvested winter rapeseed biomass. At the end of the growing season, 45% of winter oilseed rape cops contained maximum levels of metazachlor of 0.0064 mg/kg at harvest (Kucharski and Sadowski 2011). In a long-term greenhouse pot experiment, it was shown that growth and development of summer rapeseed was negatively affected when exposed to the recommended rate of 0.333 mg metazachlor kg^{-1} soil, resulting in 18% loss of yield at harvest (Baćmaga *et al.* 2014). In a comparable long-term greenhouse experiment with winter rapeseed, however, no harmful effects on growth nor on generative endpoints were observed (Zwerger and Pestemer 2000). Nevertheless, the fresh weight of non-target plants, such as oat, foxtail and pigweed were reduced, the production and viability of their seeds were not affected.

As it counts for other herbicides, the impact of chloroacetamides on surrounding fauna and flora by drainage or run-off should not be underestimated. As such, it was elucidated in a field trial that a 20 to 70% reduction of height occurred in *Prunus avium*, *Quercus robur*, *Acer pseudoplatanus* and *Fagus sylvatica* tree seedlings when treated with the recommended 1.25 kg metazachlor per hectare (kg a.i. ha^{-1}) (Willoughby *et al.* 2006). Five-week exposure to acetochlor and metolachlor (66 mg kg^{-1} soil) resulted in a reduction of growth in poplar clones (Gullner *et al.* 2001). Metazachlor induced adverse effects on the reproduction and the diversity of soil microorganisms and a decrease in soil enzyme activities (Baćmaga *et al.* 2014, Beulke and Malkomes 2001). Controlled culture experiments have shown that the biodegradation of alachlor by *Streptomycetes*-bacteria resulted in degradation products which could potentially enter the groundwater in twice as high concentrations as the maternal compound (Sette *et al.* 2004). The presence of metazachlor in water bodies has been observed in karst aquifers (with concentrations up to 82.9 ng L^{-1}) and could be linked with agricultural application events in the surrounding upstream area and by precipitation events (Hillebrand *et al.* 2014). However, higher metazachlor concentrations between 5 - 200 µg L^{-1} have been detected in streams and aquifers (Kreuger 1998, Kreuger *et al.* 1999). Downstream aquatic communities in ponds and streams were likely to be affected by 5 µg L^{-1} metazachlor, with a decrease in phytoplankton, zooplankton and macrophyte densities (Noack *et al.* 2003, Mohr *et al.* 2008). Hereby, metazachlor-induced inhibition of chlorophytes and macrophytes resulted indirectly in reduced zooplankton densities due to habitat loss. Growth of two duckweed species was strongly inhibited after one single exposure event to metazachlor concentrations that were ecologically relevant in surface waters (0.6 - 150 µg L^{-1}) (Müller *et al.* 2010). Together with other herbicides, metazachlor was found to be present in muscle tissue of fish that were naturally exposed to pond watershed pesticide pressure (Lazartigues *et al.* 2013). However, according to prediction models, biomagnification was assumed not to occur. The potential of chloroacetamide herbicides to affect human health was investigated in a cohort study following private and commercial applicators for 7 years. Researchers did not find a link between the incidence of cancer and the exposure to metolachlor (Rusiecki *et al.* 2006). However, given the widespread use of this compound and the frequent detection

in both surface and ground water, further examination of long-term health effects are recommended by these authors.

1.4. Study item: *Brassica napus*

1.4.1. Use and production, relevance as oilseed crop worldwide and in Europe

With a two-fold increase in global production over the last ten years and with an annual production of 72.5 millions tons, *Brassica napus* L. or rapeseed gained importance as an oilseed crop worldwide, with Europe (35.3%) and Asia (32.3%) as its main producers (FAOSTAT 2014). Rapeseed is cultivated for its oil-rich seeds (40-45%), which forms the second largest source of vegetable oil in the world after sunflower (USDA 2015). The applications of rapeseed oil are manifold, ranging from vegetable oil for human consumption, over press cake for animal feed to the production of biodiesel. As a food crop, rapeseed oil has a high nutritional value because of the high content of polyunsaturated fatty acids and the lipid composition. Rapeseed oil contains linoleic and linolenic acids that are members of the essential omega-6 and omega-3 fatty acid groups respectively (Gui *et al.* 2008). These are essential compounds in mammalian nutrition because of their capacity to reduce the risk of a heart disease and reduce cholesterol (Iggman *et al.* 2011, Lin *et al.* 2013). Additionally, oil and press cake contain proteins, minerals and vitamins, such as vitamin C and vitamin E, which contribute to health by their antioxidative characteristics. The residual press cake that is being produced during the extraction of the oil is used as high protein source in animal feed production (Lamont *et al.* 2005a). Non-edible parts are used as straw for cattle or added as soil amendment.

1.4.2. Cultivation of *Brassica napus*

In agricultural practices, *B. napus* is often used as an in-between crop in rotation with winter barley and winter wheat because of its capacity to promote the soil structure (Rathke *et al.* 2005). Studies have shown that cereals that are cultivated after rapeseed had a 10% yield increase in comparison to continuous cultivations of the same crop (Lamont *et al.* 2005b). Moreover, rapeseed cultivations are very valuable for bee population as they foresee a suitable foraging opportunity for beekeepers, with an average yield of up to 150 kg

honey per hectare (Lamont *et al.* 2005b). As is the case in most Northern countries, preference goes to cultivation of winter rapeseed because of its strong growth in spring (De Ceuster 2008) and because cultivation is less labour-intensive than summer rapeseed. Moreover, since the crop survives winter in a vegetative state, the soil is protected against erosion. However, rapeseed is prone to damage from pigeons, snails and frost (Lamont *et al.* 2005a). *Brassica napus* that is cultivated for food industry purposes is deficient in erucic acid and glucosinolates (double 00 breed), because of the incapacity of mammals to digest these compounds. Winter rapeseed is sown in the period between mid-August to mid-September and is harvested at June or July. Summer rapeseed is sown in mid-March or April and is harvested in August.

1.4.3. *Brassica napus* for biodiesel

To meet the European Climate Change Objectives in 2020 regarding actions against climate change and the safeguarding of energy supplies, 20% of Europe's energy production will have to be based on renewable energy. Europe will therefore expand the use of biofuels in the transport sector, in the form of biodiesel and bioethanol, with the goal of obtaining a 10% market share (Directive 2009/28/EC). In this framework, investments in biofuels increased globally after their introduction in the early ninetees. In Europe, 5% of the arable land (5.5 million hectares) was used for the cultivation of energy crops in 2008, of which 82% was used for biodiesel production from rapeseed, 11% for bioethanol production from annual crops and 4% for biogas production (Bunzel *et al.* 2014b). In order to reach the European target of 10%, 21 million hectares are needed in de EU (Bunzel *et al.* 2015). The principal share of renewable energy is represented by first-generation biofuels that are derived from edible parts of agricultural crops, such as rapeseed (47%), soy (35%), palm oil (10%), sunflower (4%), sugar beet, cereals, maize and others (4%), which together count for a global amount of 16.1 million tonnes of biodiesel produced worldwide in 2010 (Milazzo *et al.* 2013). Whereas bioethanol is derived from cereals, biodiesel is produced through fermentation, pyrolysis or transesterification of oil crops. In Europe, 80% of the biofuels that are currently consumed in the transport sector is represented by biodiesel, which is largely derived from rapeseed, and 20% is represented by bioethanol (EASAC 2012). In contrast with

the contemporary fossil fuels, biodiesel is CO_2 neutral over its entire life cycle. Moreover, biodiesel is biodegradable and leads to fewer emissions of pollutants such as CO_x, SO_x, particulate matter and unburned hydrocarbons (Yusuf *et al.* 2011). However, increased emissions of NO_x have been linked with rapeseed cultivation (Lapuerta *et al.* 2008). A life cycle assessment has pointed out that the use of rapeseed-derived oil results in a reduction of non-renewable energy (-20%), greenhouse gas emissions (-74%) and detoriation of the ozone layer (-44 %). On the other hand, acidification (+59%), eutrophication (+214%) and photochemical smog (+119%) increases (Gonzalez-Garcia *et al.* 2013). The implication of first generation biofuels introduces concerns about potential environmental impacts on, for example, water resources, soil quality and biodiversity (Bunzel *et al.* 2014b).

1.5. How do crops cope with chemical stress?

The ability of a plant to cope with chemical stress is determined by the efficiency of detoxification metabolism on the one hand, leading to direct removal of metazachlor and its metabolites, and by the indirect activity of antioxidant enzymes on the other hand, which are responsible for maintenance of the cellular redox state.

1.5.1. Detoxification metabolism

1.5.1.1. *In general*

Mainly all agricultural crops are subjected to herbicide-induced stress. A great deal of the crops' ability to withstand chemical stress has been addressed to their ability to metabolise the toxic compounds. Physiological and genetic evidences from over the past 45 years indicate that the detoxification metabolism is a major determinant in herbicide selectivity in plants (Marrs 1996, Pinto de Carvalho *et al.* 2009). Once the herbicidal compound enters the cell, the inactive compounds will be converted into active phytotoxic compounds. This process is called bioactivation and will occur prior to detoxification, which occurs in three successive phases (Figure 1.3). In order to enable herbicidal compounds to penetrate the waxy cuticle of the leaf, most compounds are formulated as inactive hydrophobic esters. Once they have entered the cytosol, herbicidal compounds undergo ester hydrolysis to reveal their biologically active acid or

alcohol groups (Cobb and Reade 2010). During the first phase of detoxification, the hydrophilic solubility of the compound is increased by the addition or revelation of chemically active groups, such as alcohol and carboxyl groups. Hereby the aromatic ring is hydroxylated by cytochrome P450 oxidase enzymes. This is an enzyme family catalysing a wide variety of mono-oxygenation reactions in plants, including chemical defence mechanisms, but also biosynthesis and catabolism of plant hormones (Mizutani 2012). After mono-oxygenation, the solubility of the herbicidal compound is increased. However, this step will not prevent damage within the plant cell, either because of the low rates of cytochrome P450 metabolism or by the phytotoxicity of the reaction products formed during this phase. During the second phase of detoxification, the altered chemical compound is conjugated to natural cell metabolites such as glutathione (GSH) and glucose (Figure 1.3). These conjugation steps are enhanced by the action of glutathione S-transferase enzymes (GST), resulting in GS-conjugates and by glucosyl and malonyl transferases, respectively. Glutathione conjugation of xenobiotics was described for the first time in plants by Frear and Swanson (1970) and is considered to be the most crucial step in the detoxification process. Differences in the spectrum of available GST isoforms present an important role in selectivity of herbicides. As the formed GS-conjugates are able to inhibit GST activity (Schröder and Collins 2002), they are actively transported into the vacuole by ATP-binding cassette transporters (Martinoia *et al.* 1993, Coleman *et al.* 1997). Once the metabolite conjugate has entered the vacuole, it will be further metabolised either by peptidases or phytochelatin synthase (Beck *et al.* 2003). When metabolised by peptidases, cysteine conjugates are formed which are often malonated or undergo transamination to yield a thiolactic acid conjugate (Lamoureux 1983). Next to its role in the formation of phytochelatin peptides in metal detoxification, phytochelatin synthase also fulfils a role in the degradation of GS-conjugates of xenobiotics, which ultimately form glutamylcysteinyl-adducts. Finally, the metabolites will either be stored in the vacuole or excreted across the plasma membrane into the extracellular matrix of the cell wall or the rhizosphere through unidirectional long-range transport (Schröder *et al.* 2007). Therefore, the vacuole may serve as an intermediary storage pool. To protect the cell from injury, all steps mentioned in the detoxification should be highly coordinated.

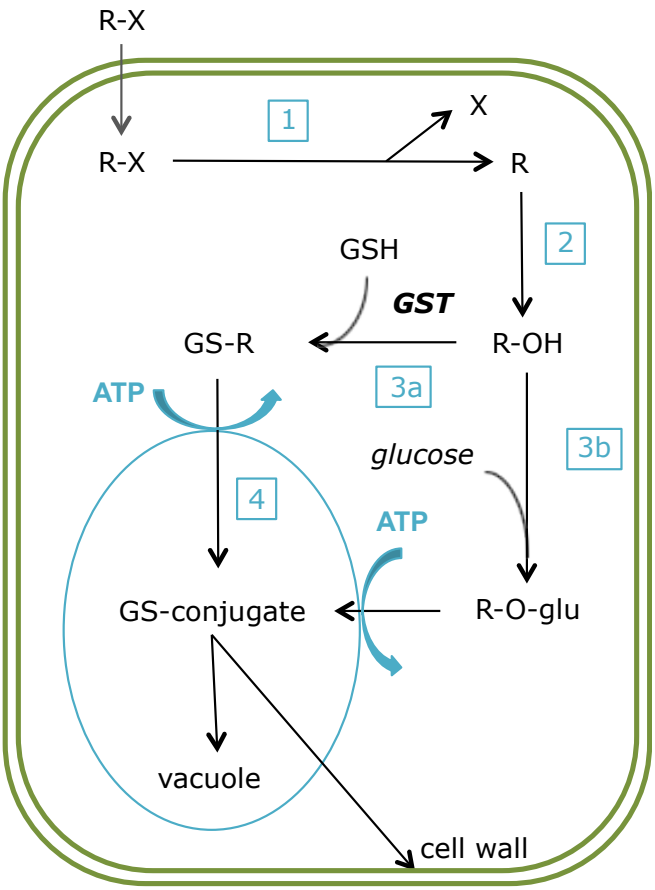

Figure 1.3. *Detoxification of xenobiotic compounds according to 4 successive steps. 1. Bioactivation. 2. Phase I – conversion by cytochrome P450. 3a Phase II – GSH conjugation, mediated by glutathione S-transferase. 3b Phase II – glucose conjugation, mediated by glucosyl and malonyl transferases. 4 Phase III – sequestration. Figure adapted from Cobb and Reade (2010).*

1.5.1.2. *Glutathione*

Glutathione (GSH) is a widely distributed tripeptide in both animals and plants. In plants cells, GSH is found at millimolar concentrations of 0.5-10 mM (Noctor *et al.* 2012). Structurally, GSH is build from three amino acids, γ-glutamate, cysteine and glycine, in two ATP-dependent steps. In the first step, a peptide bond is formed between γ-glutamate and cysteine by γ-glutamylcysteine synthetase (GSH1). Subsequently, the glutathione synthetase (GSH2)-mediated

addition of glycine results in the tripeptide GSH. Glutathione serves a variety of functions in antioxidant defence, xenobiotic detoxification, signalling and as an important storage form of reduced sulphur in cells. As an antioxidant metabolite, GSH is involved in the direct detoxification of H_2O_2, or indirectly via the AsA/GSH and the redoxin cycle (§ 1.5.2.2., Jozefczak *et al.* 2012). In the detoxification of xenobiotics, GSH serves as an important 'tag', guiding xenobiotic compounds to the vacuole for metabolisation or sequestration. The redox state of GSH has been shown to be important in determining the efficiency of conjugation with an increase in efficiency when balancing towards the reduced state (Hatton *et al.* 1996a). The diverse functions of GSH originate from the sulfhydryl group in cysteine, enabling GSH to participate in redox cycling. The principle behind the GSH conjugation lies within the reactiveness of sulphur within the peptide. Sulphur is a low charged ion, which can easily be polarised when bound. Based on this feature, sulphur forms a nucleophilic site within the GSH peptide which is able to react with a large number of xenobiotics. Hence, GSH possesses the ability to react spontaneously with low charged electrophiles (Schröder and Collins 2002). However, in order to form a bond between nucleophilic sulphur and highly charged electrophiles, this reaction requires additional enzymatic support that is foreseen by GST isozymes. In any case, detoxification is depending on the availability of reduced GSH. The homeostasis of GSH within the plant is maintained by a complex regulation process, including synthesis, degradation, long-range transport and glutathione reductase (GR) – mediated maintenance of the redox state (Alscher 2002).

1.5.1.3. *Glutathione S-transferases*

Glutathione S-transferases (GST) are predominantly expressed in the cytosol and are induced by a broad range of stressors, such as metals, ozone, drugs, herbivoric wounding and herbicides. Thereby, they are involved in both the detoxification of cytotoxic compounds and the protection of tissues against oxidative damage (Mannervik and Danielson 1988, Marrs 1996, Bartha *et al.* 2010). As a key enzyme in phase II of the detoxification metabolism in plants, the function of GST is dual. Besides the removal of harmful xenobiotic compounds, it simultaneously increases the solubility of the hydrophobic compound in the cytosol. The basis for the range of catalytic activities of GST is

its ability to lower the pK_a of the sulfhydryl group of reduced GSH from 9.0 in aqueous solution to about 6.5 when bound to the active site of the enzyme (Armstrong 1991). The electrophilic groups of a variety of xenobiotic compounds undergo a nucleophilic attack from the sulphur atom of GSH that is catalysed by GST (Karavangeli *et al.* 2005). Glutathione S-transferases that are active in herbicide metabolism in plants were described for the first time in 1970 and became a well-established determinant of selectivity in crops (Marrs 1996, Dixon *et al.* 2010). GSTs can represent more than 1% of the total soluble amount of protein in maize (Cole 1994). The high selective power of GSTs can be addressed to the physico-chemical properties of this enzyme. All plant GSTs have native molecular masses of around 50-60 kDa and are composed of two similarly sized subunits (Droog 1997, Dixon 1998). Each subunit contains a kinetically independent active site with distinct binding domains for the nucleophilic GSH and the electrophilic co-substrate, the xenobiotic compound. These subunits can be either identical resulting in homo-dimeric GSTs or distinct resulting in hetero-dimeric GSTs (Dixon and Edwards 2010). The first active site is a binding site specific for GSH or a closely related homolog formed from a conserved group of amino-acid residues in the amino-terminal domain of the tripeptide (the G site). The second active site binds the hydrophobic substrate (the H site). This site is structurally variable and is formed from residues in the carboxy-terminal domain. The majority of GST substrates are either xenobiotic compounds or products from oxidative stress. The formation of a thioether bond between electrophiles and GSH almost always yields a conjugate that is less reactive than the parental compound. GST is also suggested to be involved in protection against oxidative stress. In plants, induction of GST is due to the transient accumulation of H_2O_2 (Dudler *et al.* 1991, Hayes and Pulford 1995, Levine 1995). Hydrogen peroxide has been shown to act as the primary signal to rapidly and selectively stimulate the transcription of GST and GSH peroxidases (Marrs 1996). GST induction by ROS would appear to represent an adaptive response as these enzymes detoxify some of the toxic carbonyl-, peroxide-, and epoxide-containing metabolites produced within the cell by oxidative stress.

Plants contain complex multigene families of GSTs, with each subunit coded by a different gene. Hence, the various subunits may be able to dimerise in many

permutations, producing multiple homo- and hetero-dimeric GST isozymes in the plant. The GST isozymes involved in xenobiotic metabolism are subjected to discrete regulation, showing distinct but overlapping substrate specificities. Moreover, complementation studies have suggested that quite dissimilar GSTs share similar functions (Edwards 2000). Therefore, GST isozymes cannot be classified according to their substrate-specificity or function. Based on sequence identity and gene structure of family members, plant GST's can be divided into five classes: tau, phi, theta, zeta and lambda classes (Dixon and Edwards 2010). All classes display differences in the amino acid residues in the active site, amino acid sequence and regulation (Droog 1997). Along with the zeta class, the theta class of GSTs are conserved between plants and animals. Currently, it is known that conjugation executed by phi and tau class GSTs are the most important routes for detoxification in plants (Thom 2002). Phi class GSTs are a large, plant-specific class of proteins and are known to be involved in tolerance to oxidative stress (Sappl *et al.* 2009). Tau class GSTs are plant-specific and are the most abundant GST class in *Arabidopsis* and are found to selectively bind fatty acid derivates (Dixon and Edwards 2009). Thom *et al.* (2002) have elucidated the structural basis for herbicide binding. The structure of a tau class GST reveals a large inverted L-shaped active site, which is different in shape and size from other plant GSTs described to date. The phi class enzymes that are structurally characterized have a large rather open cavity between the G- and H-sites. The inducibility of phi and tau GSTs in plants after exposure to either biotic or abiotic stresses is a characteristic feature of these genes (Thom *et al.* 2002, Sappl *et al.* 2009). In the conjugation of chloroacetamide herbicides, tau class GSTs appear to play a role (Jo *et al.* 2011). Since the studied tau class GST displayed a high activity towards the general GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) in rice (Jo *et al.* 2011), CDNB can be regarded as a representative substrate for estimating chloroacetamide conjugation.

1.5.1.4. *Detoxification metabolism in determining herbicide selectivity*

As mentioned previously (§1.2.2.) the currently commercialised herbicidal compounds comprise a broad range of targets within the physiological processes of the plant. As such, the absence of a target in a plant species can result in tolerance against a certain herbicide (Preston *et al.* 1999, Del Buono *et al.*

2011). Besides the presence or absence of a certain target within the exposed plant, the inherent detoxification metabolism of the plant is considered as a major process underlying the tolerance of species against herbicides. Physiological and genetic evidence from over the past 45 years indicates that the principle behind herbicide selectivity in plants is the ability to metabolize and thus detoxify herbicides (Cole *et al.* 1994, Marrs 1996). Variations of individual GSTs between different cultivars can be responsible for differential herbicide tolerance (Hatton *et al.* 1996a). In addition, the relative rate of herbicide detoxification, which relies on GST activity and GSH availability, between tolerant crops and susceptible weeds can account for herbicide selectivity. Comparative studies of both crops and weeds have revealed that crops contain higher levels of detoxifying GSTs than the competing weeds (Hatton *et al.* 1999, Dixon *et al.* 2010, Del Buono *et al.* 2011). As such, wheat contained higher activities of GST within 72 hours after application than ryegrass under the influence of atrazine and fluorodifen (Del Buono *et al.* 2011). Under the influence of atrazine, metolachlor, alachlor and fluorodifen the levels of GST activity were higher in maize plants than in the competing giant foxtail in plants up to 30 days old, which resulted in higher levels of herbicide injury for the latter (Hatton *et al.* 1996a). However, the levels of available GSH were not an influencing factor in determining detoxification capacity. Furthermore, this study has shown that the importance of increased GST activity in herbicide tolerance disappeared in plants older than 30 days, indicating that plant age plays a role in determining the detoxification capacity of a plant. Hence, GSH-mediated detoxification may be more important in determining selectivity in seedlings and less important in more mature plants. In crops it was proven that an increased level of GST activity resulted in an increased tolerance against herbicides. As such, chloroacetamides were shown to enhance GST activities in monocot crops, such as maize, wheat and sorghum and in poplar (Dean 1990, Jablonkai and Hatzios 1991, Hatton 1996b, Gullner *et al.* 2001). Cottingham and Hatzios (1991) have noticed that tolerance of maize towards metolachlor was attributed to an increase of GST activity and an increase of GSH content in shoot tissue under the influence of a safener within 72 hours after application. However, the relationship between plant tolerance against xenobiotics and GSH content is not always clear. Hence, Hatton and colleagues (1996a) have stated that the redox

state of GSH might be more important in determining herbicide selectivity (or crop tolerance) than GSH availability.

1.5.2. Oxidative stress

1.5.2.1. *Pro-oxidants*

Oxidative stress refers to the state of a plant cell where the balance between pro-oxidants and anti-oxidants is disturbed in favour of pro-oxidants. A major source of pro-oxidants are reactive oxygen species (ROS), which comprise superoxide ($O_2^{\circ-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\circ}OH$) and are characterised by their highly reactive power. As such, they are able to cause structural damage to DNA, lipids, proteins and enzymes within the cell and hence disrupt cellular processes and tissues. The production of ROS is inherently present in all organisms living in aerobic environments and is produced as a by-product in normal cell metabolism. In either standard or stressed conditions ROS can originate from organelles with a highly oxidising metabolism or a marked electron flow. In chloroplasts, ROS are produced at the reaction centres of photosystem I (PSI) and photosystem II (PSII) and represent a large share of the total ROS present in the cell (Asada 2006). In addition, electron leakage at the levels of complexes I and III in the respiratory electron transport chain in mitochondria are responsible for the presence of ROS (Noctor *et al.* 2007). Additional intrinsic sources of ROS are found in peroxisomes, plasma membrane, cell wall, vacuole, endoplasmic reticulum and the cytosol (Keunen *et al.* 2013). A disturbance of the cellular redox state can be induced by external biotic and abiotic factors such as herbivores (Mithofer *et al.* 2004), light (Smirnoff 2005, Maeda and DellaPenna 2007), salinity (Yusuf *et al.* 2010), drought (Abedi *et al.* 2011), metals (Smeets *et al.* 2005, Cuypers *et al.* 2009) and herbicides (Fryer 1992) and ultimately results in sub-lethal to lethal oxidative damage. Nevertheless, ROS are also considered to conduct a key role as signalling regulators in defence pathways in order to enhance cellular protection and acclimation (Dat *et al.* 2000, Mittler *et al.* 2004). The processes determining the fine-tuning between damage and signalling are considered to occur under tightly controlled pathways.

1.5.2.2. *Antioxidative defence*

The inherent capacity of plants to cope with the imbalance of the cellular redox state, is represented by ROS-scavenging enzymes, such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APx), glutathione reductase (GR), guaiacol peroxidase (GPx), syringaldazine peroxidase (SPx), peroxiredoxines (PrxR) and antioxidative metabolites, such as ascorbate (AsA), GSH, tocopherols and carotenoids which all constitute the antioxidative defence network (Figure 1.4). Superoxide dismutase converts the superoxide ion $O_2^{\bullet -}$ into H_2O_2, which subsequently reduced into H_2O and O_2 by either CAT or APx. Catalase displays a relatively low affinity towards H_2O_2, the reduction occurs fast and in the absence of organic substrates. Peroxiredoxines neutralise H_2O_2 by the use of GSH as electron donor (Noctor 2006) (Figure 1.4). Cell wall associated peroxidases that are reactive towards guaiacol (GPx) and syringaldazine (SPx) contribute to ROS scavenging located at the cell membrane. Ascorbate peroxidase-mediated reduction of H_2O_2 is highly specific and occurs in the presence of ascorbate as electron donor. Oxidised monodehydroascorbate (MDHA) and dehydroascorbate (DHA) are subsequently reduced by reductase enzymes (MDHAR and DHAR) in the presence of GSH as electron donor. Glutathione is simultaneously oxidised into glutathione disulphide (GSSG), which in turn is recovered into its reduced state by GR. Hence, the cellular pools of AsA and GSH are maintained in their reduced state by these set of enzymes that use NAD(P)H as electron donor.

Together with AsA and GSH, tocopherols (Vitamin E) are also considered important non-enzymatic antioxidants (Prior *et al.* 2005). Whereas AsA and GSH are primary hydrophilic antioxidants able to directly neutralise ROS via the AsA-GSH cycle, tocopherol is incorporated in cell membranes and have the additional and unique ability to protect polyunsaturated fatty acid (PUFA) acyl chains from oxidative damage such as lipid peroxidation. Lipid peroxidation is caused by the action of ROS and results in the production of lipid peroxyl radicals, which can either oxidise adjoining PUFAs, leading to a chain reaction, or further react with oxygen, creating malondialdehydes (MDA) and phytoprostanes. Tocopherols can alleviate the extent of lipid peroxidation by neutralising $O_2^{\bullet -}$ and lipid peroxide radicals (Havaux *et al.* 2005, Munné-Bosch 2005). Subsequently, the

tocopheroxyl-radical that is formed by oxidation of membrane-located PUFAs is reduced by ascorbate (AsA), resulting in an interaction between three redox cylces in the antioxidative network, consisting of vitamin E, AsA and GSH (Foyer and Noctor, 2005b) (Figure 1.4). In completion, sugars and carotenoids have also been suggested to play a functional role in antioxidant defence (Cuypers *et al.* 2011, Keunen *et al.* 2013). Sugars can either act as direct scavengers of °OH (Van den Ende and Valluru 2009) or by altering gene expression and enzymatic activities of H_2O_2 detoxifying pathways (Ramel *et al.* 2009). Carotenoids are exceptional in quenching singlet oxygen at which most other phenolics and antioxidants are relatively ineffective (Prior *et al.* 2005). Moreover, they are able to convert excessive energy into heat via the xanthophyll cycle (D'Haese *et al.* 2004).

1.5.2.3. *Antioxidant potential within a species is highly correlated to herbicide stress tolerance and the occurrence of phytotoxicity*

Whereas tolerance towards herbicides has been mainly attributed to the detoxification metabolism and or alterations in the target sites of herbicides actions (Preston *et al.* 1999), it is also reported that some species are more tolerant to herbicides because of their antioxidant activities (Cummins *et al.* 1999). As such, Ma *et al.* (2013) demonstrated that the oxidative metabolism significantly contributes to mesotrione resistance in waterhemp. In line with this, Radetski *et al.* (2000) suggested that antioxidant enzyme activities are a good marker for detoxification. A comparative study between wheat and ryegrass treated with atrazine and fluorodifen showed the increment of antioxidant enzyme activities in the crop and the increment of ROS-induced lipid peroxidation the weed (Del Buono *et al.* 2011). Oxidative damage in the form of lipid peroxidation has also been recorded in ryegrass under the influence of the chloroacetamide, butachlor (Wang *et al.* 2013).

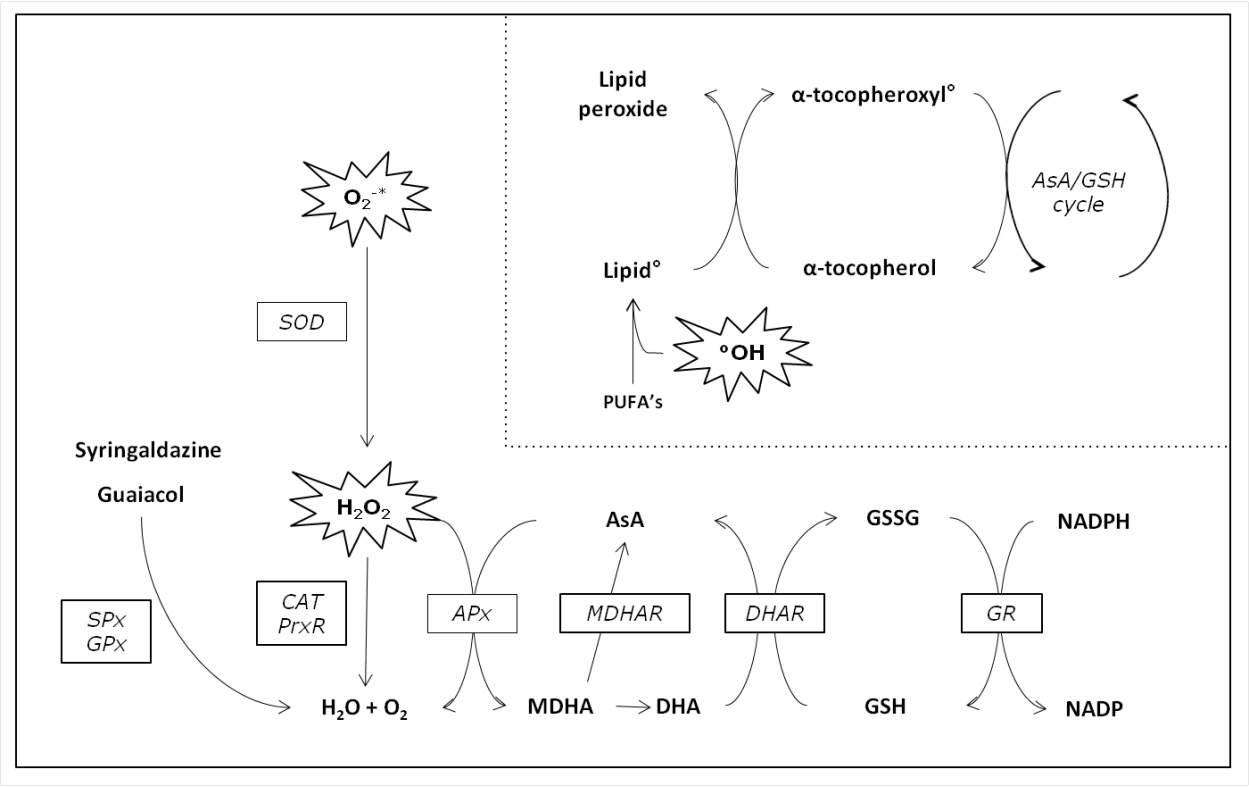

Figure 1.4. *Generalised overview of the antioxidative defence mechanism in plant cells. It should be noticed that it is divergent for different cell organelles. SOD: superoxide dismutase, CAT: catalase, APx: ascorbate peroxidase, GR: glutathione reductase, SPx:* syringaldazine peroxidase, GPx: guaiacol peroxidase, PrxR: peroxiredoxines, AsA: ascorbate , GSH: glutathione, MDHA: monodehydroascorbate, MDHAR: monodehydroascorbate reductase, DHA: dehydroascorbate, DHAR: dehydroascorbate reductase, PUFA: polyunsaturated fatty acid.

1.6. Objectives

Metazachlor is known to be an effective herbicide in winter oilseed rape (*Brassica napus*, L.) cultivations. However, it remains unclear how crops cope with this chemical stress after exposure. After all, producers of chemical pesticides use selection methods that are based on morphological endpoints, such as biomass and the appearance of chlorosis, rather than parameters concerning food quality. This study aimed at revealing (i) the occurrence of phytotoxicity in non-target, crop plants, (ii) determining the role of cellular processes involved in xenobiotic detoxification and maintenance of the cellular redox state and (iii) investigate whether short-term phytotoxicity can affect the yield and quality of *B. napus* and its end products at the end of the growing season. The objectives were tested in different experimental designs, as explained further and in Table 1.2. A detailed description of each experimental set up is given in the corresponding research chapter. Materials and methods as used in all research chapters (3 to 7) are described in **Chapter 2**.

A small-scale outdoor mesocosm experiment was conducted in plastic boxes (0.25 m^2 surface, 45 L volume) to investigate the growth and the cellular leaf status of *B. napus* one month after metazachlor application and at the end of the growing season (**Chapter 3**). The first objective in this chapter was to monitor the appearance of phytotoxic effects induced in the crop plant one month after application. The second objective was to link short-term responses to the yield and quality of *B. napus* and its end products in the long-term.

The microcosm set up (0.125 L and 1 L) enabled to zoom in on the underlying processes of the crop's ability to cope with chemically induced stress and to link the observed phytotoxic effects to cellular state (**Chapter 4**). The objective was to determine enzymes and metabolites involved in detoxification and maintenance of the cellular redox state. In accordance with the mesocosm experiment, responses were monitored at 14 and 28 days after treatment.

To investigate the rate at which the underlying mechanisms involved in herbicide detoxification and maintenance of the cellular redox state were activated, metazachlor was applied in a post-emergent way and acute responses were monitored within 24 and 72 h after application (**Chapter 5**). Additionally, a controlled semi-hydroponic experiment was set up to focus on the role of the photosynthetic apparatus in herbicide-induced stress conditions (**Chapter 6**).

A field trial with plots of each 16 m^2 was set up to investigate whether the patterns observed from the mesocosm experiment could be extrapolated to the field situation (**Chapter 7**). The second objective was to link short-term responses to the yield and quality of *B. napus* and its end products in the long-term. To conclude, an overall discussion is given and future outlooks are revealed in **Chapter 8**.

Table 1.2. *Overview of the test-systems used.*

Type experiment	Duration	Application	Doses	Sampling	Picture of set-up
Mesocosm (Chapter 3)	September-June	Pre-emergent	0, 0.75 and 1.25 kg ha^{-1}	28 days after treatment (DAT) and at harvest	
Microcosm (Chapter 4)	4 and 9 weeks	Pre-emergent	0, 0.2, 0.4 mM	14 and 28 days after treatment (DAT)	
Microcosm (Chapter 5)	2 and 9 weeks	Post-emergent (14-day-old seedlings)	0, 0.2, 0.4 mM	24 and 72 hours after treatment (HAT)	
Hydroculture (Chapter 6)	4 weeks	Pre-emergent	0, 0.25, 0.50, 0.75 and 1.00 kg ha^{-1}	28 days after treatment	
Field (Chapter 7)	September-June	Pre-emergent	0, 0.75, 1.25 and 2.0 kg ha^{-1}	At harvest	

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Chapter 2 Materials and methods

2.1. Treatment of metazachlor in different test systems

The recommended regulations concerning the application of metazachlor on oilseed rape (*B. napus*) cultivations range between 1.5 to 2 L of commercialised product (Butisan S) per hectare (BASF, Fytoweb). Since this product has a concentration of 0.5 kg L^{-1} active ingredient, these recommended doses correspond with 0.75 to 1.0 kg of active ingredient per hectare (kg a.i. ha^{-1}). Since 2013, the maximum application dose is set on 2 L per hectare (Fytoweb). According to the guidelines foreseen by the manufacturer, Butisan S is adviced to be dissolved in a minimal volume of 1000 L of water per hectare (Edialux, 2014), which corresponds with concentrations of 2.7 to 3.6 mM metazachlor per plant (Table 2.1). In practice however, farmers tend to use a higher volume (2.5 L) at their oilseed rape cultivations (Personal communication). In addition, these doses are often dissolved in a smaller volume of 400 L water due to practical constraints of limited tank volumes, which results in doses that are above the maximum advised dose, ultimately ranging around 11.25 mM per plant (Table 2.1). For the conduction of a controlled mesocosm experiment (Chapter 3), we have opted to test both the application dose as it was recommended in before 2013 and the dose often used by farmers in practice, which correspond with 0.75 and 1.25 kg a.i. ha $^{-1}$, respectively (Table 2.1). These doses were applied according to similar concentrations used in practice (3.6 and 11.25 mM, respectively). In both microcosm experiments (Chapter 4 and 5), the applied concentrations were based on the recommended concentration of 1.0 kg a.i. per hectare, dissolved in 1000 L of water (Table 2.1). Since a sandy substrate was used in these test systems, metazachlor was highly bio-available to the plants. Therefore, doses were diluted 10 times and 20 times, resulting in 0.36 and 0.18 mM, which corresponded with 1.0 mg and 0.50 mg of metazachlor per plant. Likewise to the microcosm experimental design, the hydroponics experiment (Chapter 6) offered the opportunity to solely test metazachlor-induced phytotoxicity in a one-factorial test set-up. In this study, the highest applied metazachlor concentration was based on the concentration used by farmers in practice, which corresponded with 11.25 mM metazachlor (Table 2.1). Since the latter concentration was lethal for the plants in this hydroponic test system, the following concentration range was selected: 0, 2.25, 4.50, 6.75 and 9.00 mM which corresponded with 0, 0.25, 0.50, 0.75 and 1.0 kg active ingredient per

Table 2.1: *Overview of the concentrations of metazachlor applied in different experimental test-set-ups.*

Chapter	Experiment	Dose	a.i. ha^{-1}	V (solution)	mM
		Adviced (<2013)	0.75 kg	1000 L	2.70
		Adviced (>2013)	1.00 kg	1000 L	3.60
		In practice (1000 L)	1.25 kg	1000 L	4.50
		In practice (400 L)	1.25 kg	400 L	11.25
		Based on dose used as ...	**a.i. ha^{-1}**	**V (solution)**	**mM**
3	**Mesocosm**				
		Adviced (<2013)	0.75 kg	1000 L	2.70
		In practice (400 L)	1.25 kg	400 L	11.25
			a.i. ha^{-1}	**a.i./plant**	**mM**
4, 5	**Microcosm**	Advised (>2013)	1.00 kg	1.0 mg	3.60
				1.0 mg	0.36
				0.5 mg	0.18
				0 mg	0
			a.i. ha^{-1}	**V (solution)**	**mM**
6	**Hydroponics**	In practice (400 L)	1.25 kg	400 L	11.25
			1.00 kg	400 L	9.00
			0.75 kg	400 L	6.25
			0.50 kg	400 L	4.50
			0.25 kg	400 L	2.25
			0 kg	400 L	0
			a.i. ha^{-1}	**V (solution)**	**mM**
7	**Field**		2.00 kg	400 L	18.00
		In practice (400 L)	1.25 kg	400 L	11.25
			1.00 kg	400 L	9.00
			0 kg	400 L	

hectare (kg a.i. ha^{-1}). The metazachlor doses that were tested in the long-term field experiment (Chapter 7) were based on the concentrations used in practice, by farmers (Table 2.1). Two additional doses were tested during this experiment, one was a substantial higher dose of 2.0 kg kg a.i. ha^{-1} and one was a lower dose of 1.0 kg a.i. ha^{-1}.

2.2. Morphological measurements

Stem height was determined from the transition zone between root and shoot up to the apical meristem of the plant. Fresh biomass was determined directly after sampling at a microbalance. Dry weight was determined by drying the fresh biomass in a 30°C stove for several weeks. The development of the plants was followed up by determination of the developmental stage as described by Lancashire *et al.* (1991) (Supplementary file A).

2.3. Lipid peroxidation

Lipid peroxidation of cell membranes was determined according to Dhindsa *et al*. (1981) and expressed as thiobarbituric acid (TBA) reactive metabolites. Fresh leaf tissue (100 mg) was homogenised in 0.1% trichloroacetic acid (TCA). After 10 min of centrifugation at 20,000 *g* (4°C), the extract was incubated with 0.5% TBA in 20% TCA at 95°C for 30 min. Hence, the extract was cooled for 5 min on ice (4°C) and subsequently centrifuged for 10 min at 20,000 *g* (4°C). The absorbance of the supernatant was measured at 532 nm and corrected for unspecific binding at 600 nm (UV-1800, Shimadzu, Japan).

2.4. Potassium leakage

Potassium leakage was monitored as a measure for cell membrane integrity. After cutting a leaf, the surface of the leaf was washed with Milli-Q water, dried and subsequently cut in 2 halves. Thereby, the main leaf vein was removed. After weighing each half of the leaf, one part was incubated in 10 mL of Milli-Q water at 4°C during 3 hours and the other part was incubated in10 mL of Milli-Q water at 95°C during 3 hours. The concentration of potassium was determined in both extracts by ICP-OES and represented the extracellular and the total concentration of potassium present in the leaf, respectively.

2.5. Pigment profile

Chlorophyll *a*, chlorophyll *b* and carotenoid concentrations were determined according to Lichtenthaler *et al.* (2005). Fresh leaf material (100 mg) was homogenised in 80% aceton in cooled mortars, in darkness. After centrifugation (9,000 *g*, 5 min), the volume of the supernatant was determined and subsequently 10 times diluted in 80% aceton. The leaf extract was measured spectrophotometrically (UV-1800, Shimadzu, Japan) at 663 nm, 646 nm and 470 nm, and subsequently the pigment profile was calculated.

2.6. H_2O_2 quantification

The presence of H_2O_2 in the first leaf pair was determined by qualitative 3,3'-diaminobenzidine (DAB) staining (Daudi *et al.* 2012). Since DAB precipitates as a brown complex after being oxidised by H_2O_2, the latter could be located visually. Leaves were carefully cut at their basis, put in the dark and immediately infiltrated with DAB in 10 mM Na_2HPO_4 buffer (pH 3) for 5 min under vacuum conditions. Subsequently the samples were shaken for 4 h at 80 rpm in dark conditions. After being bleached for 15 min in ethanol:acetic acid:glycerol (3:1:1) at 95 °C, leaves were stored in acetic acid (20%) at 4 °C before being monitored. The following day, detailed close-up pictures were taken from each separate leaf using a binocular microscope, a digital camera and BTV-pro software (Bensoftware).

2.7. Total antioxidant capacity

The ferric reducing antioxidant power (FRAP) assay was used to determine the capacity of lipophilic and hydrophilic antioxidant fractions (Kerchev and Ivanov 2008). Fresh leaf tissue (100 mg) was homogenised in 0.01 N Na-EDTA. After centrifugation (30 min, 15,000 *g*, 4 °C), the hydrophilic fraction was located in the supernatant. The lipophilic fraction, which was located in the pellet, was further extracted for 2 h in 80% acetone before analysis. Freshly prepared FRAP reagent, containing 100 mM 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) and 200 mM $FeCl_3$ in sodium acetic buffer (pH 3.6-4), was added to both fractions. The measurement of the antioxidative capacity of the sample was based on its ability to reduce the yellow-coloured Fe^{3+}-TPTZ complex to the blue-coloured ferrous form, which was spectrophotometrically recorded at 593 nm. The results were

calculated by standard curves prepared with known concentrations of Trolox and were expressed as µmol Trolox equivalents g^{-1} FW.

2.8. Antioxidant enzyme activities

Proteins were extracted from leaf samples by a two-step ammonium sulphate precipitation method. All steps were performed at 4°C. Leaf material was incubated for 30 min in fresh 0.1 M Tris/HCl buffer (pH 7.8), containing 5 mM EDTA, 5 mM DTE, 1% PVP and 1% Nondidet. After 30 min of centrifugation (50,000 *g*), the supernatant was incubated for 30 min with 40% $(NH_4)_2\ SO_4$. After a second round of centrifugation, the supernatant was incubated for 30 min with 80% $(NH_4)_2\ SO_4$. The extract was subsequently desalted by running over PD 10 columns (2 min, 950 *g*, 4°C) and directly stored at -80°C for further analysis of SOD, CAT, GR, SPx, GPx and GST activities. All enzyme activities were determined at 25°C in 1 mL cuvettes. Eight biological replicates were used from each condition.

Superoxide dismutase (SOD) activity was determined in 33 mM KH_2PO_4 reaction buffer (pH 7.8) and 0.1 mM EDTA (McCord and Fridovich 1969). By adding 60 mU xanthine oxidase to 0.05 mM xanthine, uric acid is formed. In this reaction, superoxide is formed as a by-product and reduces cytochrome C (0.01 mM) in a blank sample. By adding plant extract, SOD activity is calculated indirectly by measuring the inhibition of formation of reduced cytochrome C at 550 nm. The amount of SOD inhibiting the production of reduced Cyt C with 50% is defined as 1 unit of SOD activity.

Catalase (CAT) activity was determined in 75 mM KH_2PO_4 reaction buffer (pH 7) (Bergmeyer *et al.* 1974). After addition of 1 mM H_2O_2, catalase activity is calculated by the rate at which H_2O_2 is reduced to H_2O and O_2 and hence by measuring the decrease of H_2O_2 spectrophotometrically at 240 nm.

Glutathione reductase (GR) was determined in 1 mM Tris and 1 mM EDTA reaction buffer (pH 8) (Bergmeyer *et al.* 1974). By adding 1.5 mM glutathione disulfide (GSSG) and 0.1 mM NADPH to the reaction buffer, GR present in the leaf extract catalyses the reduction of GSSG to GSH, through simultaneous

consumption of NADPH. Measuring the oxidation of NADPH at 340 nm makes it possible to calculate the activity of GR.

Guaiacol peroxidase (GPx) was determined in 75 mM KH_2PO_4 reaction buffer (pH 7) (Bergmeyer *et al.* 1974). After the addition of 1 mM H_2O_2 and 2 mM guaiacol to the reaction buffer, the subsequent addition of leaf extract catalysed the conversion of H_2O_2 into H_2O and O_2 by oxidation of guaiacol, which was measured spectrophotometrically at 436 nm.

Syringaldazine peroxidase (SPx) was determined in 80 mM Tris-HCl reaction buffer (pH 7.5) (Czaninski *et al.* 1984). Syringaldazine substrate (55 µM) was oxidised by SPx simultaneously with the reduction of 1 mM H_2O_2 and was monitored at 530 nm.

Glutathione S-transferase (GST) activity was determined using different standard substrates: 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), 1 mM 1,2-dichloro-4-nitrobenzene (DCNB), 1 mM 4-nitrobenzyl chloride (NBC), 1 mM p-nitrobenzoyl chloride (NBoC), 0.5 mM p-nitrophenylacetaat (p-Npa) and fluorodifen (1.2 mM) (Scalla and Roulet 2002, Schröder and Collins 2002). By adding 1 mM glutathione (GSH) to 1 mM of substrate in 75 mM KH_2PO_4 reaction buffer (pH 6.5), the formation of conjugate was measured at respective wavelengths (340 nm, 345 nm, 310 nm, 310 nm, 400 nm and 400 nm).

Ascorbate peroxidase (APx) activity was determined after a separate extraction (Gerbling *et al.* 1984). Plant tissue (100 mg) was extracted using a modified extraction buffer, containing 0.1 M Tris-HCl (pH 7.8), 1 mM DTT, 1 mM EDTA and 10 mM ascorbate. Ascorbate peroxidase reduces H_2O_2 by oxidation of ascorbate (AsA) into dehydroascorbate (DHA). Adding 20 mM H_2O_2 to the reaction buffer (0.1 M Hepes and 1 M EDTA, pH 7), made it possible to calculate APx activity in leaf extract by monitoring the decrease of ascorbate (AsA) at 298 nm.

2.9. Metabolite concentration and redox state

Leaf tissue was extracted in 200 mM HCl. After centrifugation (16,000 *g*, 10 min, 4°C), the supernatant was diluted with 200 mM NaH_2PO_4 (pH 5.6) and brought to pH 4.5 by addition of 200 mM NaOH. This extract was used for the determination of both AsA and GSH concentrations and their redox state (Queval and Noctor 2007). Ascorbate determination was based on ascorbate oxidase (AO) mediated oxidation of AsA. Dithiotreitol (DTT, 25 mM) was added to one half of the leaf extract, reducing all present DHA. By addition of AO to the subsample without DTT and the subsample with DTT, the reduced fraction (AsA) and the total fraction of ascorbate (AsA and DHA) could be determined, respectively, by spectrophotometric measurement of the decrease in reduced ascorbate at 265 nm. Glutathione measurement was based on GSH mediated reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) that was analysed using a spectrophotometer at 412 nm. By incubating half of the plant extract with 2-vinylpiridine for 30 minutes at 20°C, GSH was inactivated and only the present oxidised fraction of glutathione (GSSG) could be measured. By addition of GR to both incubated and non-incubated subsamples, GSSG and total GSH concentration could be determined by monitoring the reduction of DTNB by GSH.

2.10. Metazachlor determination in leaves

Metazachlor was determined in the aboveground biomass via reverse-phased high performance liquid chromatography (RP-HPLC) (Adept CE-4200, Dual Piston Pump CE 4120, UV/VIS detector and Power Stream software, United Kingdom). For extraction, 5 g of fresh plant tissue was homogenised in 5 mL pure acetonitrile (HPLC grade JTBaker ®). To improve the recovery of polar components and to facilitate the partitioning of the solvent, 2 g $MgSO_4$ was added to the extract. In order to reduce the amount of polar interferences 0.5 g NaCl was added. By the addition of 0.5 g Na_3 Citrate x 2 H_2O and 0.25 g Na_2H Citrate x 1.5 H_2O, the optimal pH of 6.5 was maintained. The extract was centrifuged for 5 min at 1,057 *g* at room temperature. One mL of supernatant was transferred to a dispersive centrifuge tube (Spectrum ®, Chemical MFG Corp.) containing 25 mg of primary secondary amine and 150 mg $MgSO_4$ and 2.5 mg of graphitised carbon black to remove pigments. The tubes were mixed for 30 s and then centrifuged again for 5 min at 1,057 *g*. Hence, the collected

supernatant was analysed by RP-HPLC. The samples were analysed at 220 nm wavelength in a gradient regime by an analytical column Supelcosil LC-18 150 x 4.6 mm, 5 µm. The injected volume was 20 µL. The mobile phase composition was: phase A acetonitrile: water (40:60) and phase B acetonitrile: water (80:20). The gradient was applied for 20 min in the following regime; 0 min - 100% A**:** 0% B, 10 min - 50% A**:** 50% B, 20 min - 0% A: 100% B, with a flow rate of 1 mL/min. The limit of quantification and the limit of detection of this analytical method contained 1.1 µg/mL and 0.4 µg/mL, respectively and 85% recovery. Quantification was based on a metazachlor standard curve prepared with certified metazachlor standard (98.5%, Dr Ehrenstorfer GmbH).

2.11. Nutrient profile in leaves and seeds

Dry leaf material (0.1-0.5 g) was digested in 70-71% HNO_3 and dissolved in 2% HCl. Seeds (0.5 g) were digested for extraction in 65% HNO_3 and 30% of H_2O_2 in a MLS-1200 Mega microwave (Milestone Laboratory Systems, Italy) according to the following heating cycle: 1 min at 250 W for, 1 min at 0 W, 5 min at 250 W, 6 min at 400 W and 5 min at 650 W. After digestion, the clear colourless extract was brought to 25 mL volume with Milli-Q water. Macronutrients (P, K, Mg, Ca, S) and micronutrients (Na, Fe, Cu, Zn, Mn) present in plant extracts were determined by inductively coupled plasma – optical emission spectroscopy (ICP-OES 710, Agilent Technologies, Australia). Concentrations were calculated by the use of standard curves with known concentrations.

2.12. Fatty acid profiling

Fatty acids were determined according to ISO norm 12966-2 (2011). Seeds (0.4 g) were crushed and oil was extracted by homogenization in 5 mL of iso-octane, and subsequent centrifugation (3 min, 1,000 *g*). Lipids obtained from extraction were subsequently converted to their corresponding fatty acid methyl esters (FAMEs) by trans-esterification with potassium hydroxide (2 M). Two mL of the extract was homogenised by vortexing with 100 µL of methanolic potassium hydroxide (2 M) during 30 sec. The solution was neutralised by the addition of 0.5 g $NaHSO_4$ (H_2O). After the salt was settled, 1 mL of the upper phase was transferred into a 2 mL vial for gas chromatography (GC Trace 2000 ThermoQuest, ThermoFinnigan, Milan, Italy). The fatty acids were analysed on a DB-WAX capillary column (Agilent 30 m x 0.25 mm). The injected volume was

1 µL and the temperature of the injector was 250 °C. Helium was used as a carrier gas at a constant flow of 1 mL/min. The oven had a constant temperature of 220 °C.

2.13. Flavonoid profiling

From each condition, 30 seeds were weighed in an Eppendorf tube. After addition of three stainless beads, 1.5 mL methanol and 1% formic acid, the seed samples were crushed. Subsequently, 1.5 mL methanol and 1% formic acid were added and homogenised by vortexing. After 10 min in an ultrasonic bath, samples were briefly centrifuged (1 min at 1,000 *g*). Before injection, 900 µL of this extract was filtered with a Teflon filter syringe (diameter 13 mm). Chromatographic separation was carried out on an UPLC-MS system (Waters Acquity TQD, Belgium), which is equipped with a C18 column BEH (2.1 x 150 mm, 1.7 µm, Waters ®) and a photodiode array detector (PAD) that scans from 190 nm to 600 nm. The injected volume of each sample was 2 µL. The composition of the mobile phase was A: Ultra purified water and B: Acetonitrile + 0.1 % formic acid. A gradient was applied for 10 min starting with 98% A: 2% B at 0 min – 38% A: 62% B at 3 min – 10% A: 90% B at 8min – 98% A: 2% B at 10 min. Quantification of flavonols was based on PDA measurement of external standard solutions of known flavonols. The mass spectrophotometer was used for identification of flavonols that were not present in the standard solution.

2.14. Statistical analyses

Data were processed according to one-way or two-way ANOVA tests in open-source R software (R 3.1.2, The R Foundation for Statistical Computing, Vienna), in strict accordance to parametrical conditions. Normal distribution of the data was tested using Shapiro-Wilk test. Following the ANOVA, post-hoc Tukey test was performed for multiple pairwise comparisons. In case parametrical conditions were not met, Kruskall-Wallis, followed by two-by-two Wilcoxon post-hoc comparison analyses were performed. Data are represented as mean values ± standard error (SE) ans significance was set at 5% level. Asterisks represent significant differences of a treatment in comparison with the control value.

2.15. References

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2.16. Supplementary files

Supplementary file A. *Phenological growth stages and BBCH-identification keys of Brasscica napus L. ssp. napus, adapted from Weber and Bleiholder (1990) and Lancashire et al. (1991).*

Code	Description
Principal growth stage 0: Germination	
0	Dry seed
1	Beginning of seed inbibition
3	Seed inbibition complete
5	Radicle emerged from seed
7	Hypocotyl with cotyledons emerged from seed
8	Hypocotyl with cotyledons growing towards soil surface
9	Emergence: cotyledons emerge through soil surface
Principal growth stage 1: Leaf development	
10	Cotyledons completely unfolded
11	First leaf unfolded
12	2 leaves unfolded
13	3 leaves unfolded
14	4 leaves unfolded
15	5 leaves unfolded
16	6 leaves unfolded
17	7 leaves unfolded
18	8 leaves unfolded
19	9 or more leaves unfolded
Principal growth stage 2: Formation of side shoots	
20	No side shoots
21	Beginning of side shoot development: first side shoot detectable
22	2 side shoots detectable
23	3 side shoots detectable
24	4 side shoots detectable
25	5 side shoots detectable
26	6 side shoots detectable
27	7 side shoots detectable
28	8 side shoots detectable
29	End of side shoot development: 9 or more side shoots detectable

Code	Description
Principal growth stage 3: Stem elongation	
30	Beginning of stem elongation: no internodes ("rosette")
31	1 visibly extended internode
32	2 visibly extended internodes
33	3 visibly extended internodes
34	4 visibly extended internodes
35	5 visibly extended internodes
36	6 visibly extended internodes
37	7 visibly extended internodes
38	8 visibly extended internodes
39	9 or more visibly extended internodes
Principal growth stage 5: Inflorescence emergence	
50	Flower buds present, still enclosed by leaves
51	Flower buds visible from above ("green bud")
52	Flower buds free, level with the youngest leaves
53	Flower buds raised above the youngest leaves
55	Individual flower buds (main inflorescence) visible but still closed
57	Individual flower buds (secondary inflorescence) visible but still closed
59	First petals visible, flower buds still closed ("yellow bud")
Principal growth stage 6: Flowering	
60	First flowers open
61	10% of flowers on main raceme open, main raceme elongating
62	20% of flowers on main raceme open
63	30% of flower on main raceme open
64	40% of flowers on main raceme open
65	Full flowering: 50 % flowers on main raceme open, older petals falling
67	Flowering declining: majority of petals falling
69	End of flowering
Principal growth stage 7: Development of fruit	
71	10% of pods have reached final size
72	20% of pods have reached final size
73	30% of pods have reached final size
74	40% of pods have reached final size
75	50% of pods have reached final size
76	60% of pods have reached final size
77	70% of pods have reached final size

Code	Description
78	80% of pods have reached final size
79	Nearly all pods have reached final size
Principal growth stage 8: Ripening	
80	Beginning of ripening: seed green, filling pod cavity
81	10% of pods ripe, seeds dark and hardRipening
82	20% of pods ripe, seeds dark and hard
83	30% of pods ripe, seeds dark and hard
84	40% of pods ripe, seeds dark and hard
85	50% of pods ripe, seeds dark and hard
86	60% of pods ripe, seeds dark and hard
87	70% of pods ripe, seeds dark and hard
88	80% of pods ripe, seeds dark and hard
89	Fully ripe: nearly all pods ripe, seeds dark and hard
Principal growth stage 9: Senescence	
97	Plant dead and dry
99	Harvested product

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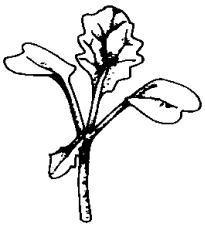

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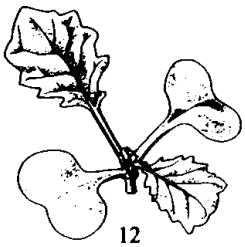

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Chapter 3 Short- and long-term responses of oilseed rape (*Brassica napus* L.) to metazachlor: a mesocosm study

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3.1. Abstract

Two semi-controlled mesocosm experiments were set up to investigate the relation between the initial responses of the oilseed rape crop (*Brassica napus* L.) to the herbicide metazachlor, and how this pre-emergent treatment can influence the crops' final end products at harvest time. This study elucidated that when seeds are sown in excess, plants suffer more from intraspecific competition than chemical stress, shortly after herbicide treatment. However, when intraspecific competition between crop plants was excluded, symptoms of metazachlor-induced stress became more obvious in the short-term. Lowered seed emergence, stem length, biomass and pigment content were observed in metazachlor-treated plants together with the appearance of fasciation. At the cellular level, no effect on herbicide detoxification via glutathione S-transferase (GST) was noticed, but metazachlor-induced oxidative stress was clearly present. More specifically, an increased activity of hydrophilic antioxidant enzymes and metabolites, a decrease in lipophilic antioxidants and a shift in the ascorbate redox state were observed one month after metazachlor application. Yet, at the long-term, oilseed rape recovered from these initial symptoms resulting in biomass production and oil characteristics equivalent to non-treated plants. In conclusion, this study elucidates for the first time that the use of metazachlor significantly affects the growth and cellular responses of the plants in a controlled mesocosm experiment. In addition, in order to cope with the herbicide-induced stress, plants activate their cellular antioxidative defence mechanisms.

3.2. Introduction

Since the interbellum, the agricultural sector is increasingly relying on the use of synthetic pesticides in order to ensure food and feed production (Cobb and Reade 2010, Costa 1987). From then on, pesticides that act on a broad range of target organisms have been developed, commercialised and applied on arable lands, thereby preventing crop injury induced by unwanted insects, fungi, weeds and bacteria (Cooper and Dobson 2007, Zhang *et al.* 2009). Herbicides, which represent the largest subgroup of pesticides, comprise all chemical molecules that act specifically on plant functional processes and pathways such as photosynthesis, biosynthesis of amino acids and proteins, thereby inhibiting

weed growth (Bijanzadeh *et al.* 2010, Cobb and Reade 2010, Retzinger and Mallory-Smith 1997). Worldwide, approximately one billion kilograms of herbicidal active ingredients (a.i.) are applied on crops, in accordance with a market share of 16 billion dollars in 2007 (Grube *et al.* 2011). Prudence requires us to take into account adverse effects of herbicides on crops and their plant-associated bacteria and bacterial communities and enzymes present in the soil as is indicated in several studies (Baćmaga *et al.* 2014, Beulke and Malkomes 2001, Muñoz-Leoz *et al.* 2012, Zaidi *et al.* 2005). When exposed to herbicides in either pre- or post-emergent application, crops can undergo changes in their pigment profiles (Alonge 2000, Carpenter and Boutin 2010) and photosynthetic activities (Jursík *et al.* 2013) leading to yield loss (Gilreath *et al.* 2001, Majid *et al.* 2003, Young *et al.* 2003).

Brassica napus is an important crop in North America, Europe (especially France and Germany) and Asia. The end products of rapeseed are oil, which can be used for human consumption as well as biofuel applications, and press cake that can be used as feed for animals. Metazachlor is a pre-emergent applied herbicide that is specifically used on winter oilseed rape (*B. napus*) cultivation, worldwide (European Commision 2012). Metazachlor belongs to the chemical group of chloroacetamide herbicides, which are characterised by their ability to interfere with CoA-enzyme function and consequently inhibit very long chain fatty acid (VLCFA) biosynthesis (Böger 2003). Metazachlor binds irreversibly to the CoA-enzyme synthase and cannot be displaced by the substrate fatty acids (Götz and Böger 2004). In addition, the fatty acid substrates are easily removed from the CoA-enzyme by metazachlor. Consequently, flavonoid and anthocyanin biosynthesis are affected by chloroacetamides, which can lead to impaired membrane formation and altered lipid biosynthesis (Böger 2003).

The fate of chemicals applied is manifold and subsequently imposes indirect risks to the ecosystem and human health (Claeys *et al.* 2011, Marrs 1996, Wilson and Tisdell 2001). Through drainage and run-off, pesticides and their derivatives can enter the groundwater and the water cycle in general, therefore becoming bio-available via drinking water and in the meanwhile leading to negative effects in aquatic ecosystems by inhibiting macrophyte and plankton

growth *e.g.* at concentrations above 5 µg/L metazachlor (Mai *et al.* 2013, Mohammad *et al.* 2008, Mohr *et al.* 2008). Furthermore, when persistent in soil and plants, herbicidal molecules and their derivatives can enter the food chain. Oosterhuis *et al.* (2008) have shown high affinity of chloroacetamide herbicides with the human MDR1 transporter, thereby affecting intestinal drug absorption. Whereas guidelines and criteria for the use of herbicides have been set by the Organisation for Economic Co-operation and Development, little is known about the consequences of pre-emergent application of herbicides towards the quality of the end products of crops (OECD 2009). When pre-emergently applied, herbicides do not only inhibit the appearance of unwanted weeds, they also come into contact with sown crop seeds. Besides physiological effects, herbicides can also induce formation of reactive oxygen species (ROS) at the cellular level, leading to disruption of cellular structures and processes. Previous studies illustrated the induction of antioxidative enzymes and metabolites in response to herbicide application (Cui *et al.* 2010, Ivanov *et al.* 2005, Jiang *et al.* 2010). The degree of phytotoxicity is also determined by glutathione S-transferase-(GST)-mediated detoxification of the herbicide. GSTs are crucial enzymes in xenobiotic detoxification processes. Through their ability to conjugate GSH to the herbicide, the resulting conjugates are easily neutralised by uptake in the vacuole or incorporation in cell membranes (Dixon *et al.* 1998, Karavangeli *et al.* 2005). The occurrence and extent of herbicide-induced phytotoxicity are determined by the rate and efficiency of detoxification on the one hand, leading to direct removal of metazachlor and its metabolites. On the other hand, the induction of antioxidative enzyme activities is an indirect measure of phytotoxicity as they are responsible for reducing the herbicide-induced ROS production.

In this study a semi-controlled experimental set-up in mesocosms was used to study (i) whether the pre-emergent application of metazachlor resulted in short-term phytotoxicity in *B. napus* at different levels, (ii) which plant mechanisms play a key role in determining the extent of phytotoxicity and (iii) if short-term phytotoxicity is reflected in the vegetative and regenerative end products of *B. napus* in the long-term.

3.3. Experimental design and methodology

The study site was located in Diepenbeek (Belgium). Plastic mesocosms with 0.25 m^2 surface area and 25 cm depth were dug in at ground level. Soil was collected from a cultivated farmland and mixed homogenously before being added to the mesocosms. Drainage was foreseen by holes in the bottom of the mesocosms covered with drainage grains and an anti-rooting membrane, the latter preventing the roots growing through the holes in the bottom. Two experiments were carried out, in two successive growing seasons from 2012 to 2014. The experimental design and the soil properties of both experiments are shown in supplementary file A. The main difference between the two experimental set-ups lies in the amount of seeds that were sown per mesocosm. In both experiments, seeds of winter oilseed rape (*Brassica napus* L., var. *Remy*) were sown in the first week of September. In the first experiment, seeds were sown in a three-times higher abundance as recommended at 1 cm below soil surface. One month after sowing, plants in control plots were thinned out to a final amount of approximately 30 plants per mesocosm. Simultaneously, weeds were removed in this treatment. In the second experiment, a fixed amount of 36 seeds was sown at 1 cm below soil surface. One month after sowing, weeds in control treatments were manually removed. In both experiments, metazachlor (Butisan S, 500 g L^{-1} metazachlor, BASF plc.) was applied by spraying on the soil surface one day after sowing in a recommended concentration of 0.75 kg active ingredient per hectare (a.i. ha^{-1}) and according to a more concentrated dose of 1.25 kg a.i. ha^{-1}, often used in practice by farmers (personal communication). All treatments (0, 0.75 and 1.25 kg a.i. ha^{-1}) were applied in 6 to 8 replicate mesocosms, according to a randomised block design.

In the first experiment, 16 plants were selected from each condition for stem length measurement and for the determination of their developmental stage (Lancashire *et al.* 1991). In both experiments, morphological characteristics, such as aboveground fresh weight and stem length, were determined at 28 DAT (days after treatment). Simultaneously, fresh tissue of the first leaf pair was collected, snap-frozen and immediately stored at -80°C prior to biochemical analyses. Membrane lipid peroxidation, pigment profile, nutrient profile, total

antioxidant capacity, H_2O_2 concentration, activity of antioxidative enzymes and redox state of antioxidant metabolites, AsA and GSH, were determined at this time point. At the end of June, the full-grown oilseed rape plants were harvested for height and fresh and dry weight determination. Life history parameters, such as seed yield per plant and seed weight, were determined. Nutrient profiles of aboveground biomass and of seeds were determined, together with the fatty acid profile and the presence of flavonoids in the oil.

3.4. Results

3.4.1. Intraspecific competition over herbicidal stress

3.4.1.1. *Short-term data*

Immediately after the onset of the first experiment, in which seeds were sown abundantly and mesocosm plots were thinned out one month after sowing, germination of *B. napus* was adversely influenced by herbicide treatment with metazachlor. Plots treated with 0.75 and 1.25 kg a.i. ha^{-1} metazachlor contained less seedlings than control plots, nine days after treatment (9 DAT) (Figure 3.1a). Moreover, seedling cotelydons in these plots were smaller in size at this time point. Since seeds were sown in excess, germination capacity could not be determined quantitatively in control plots. The initial stem length of non-treated seedlings at 9 DAT was 1.8 and 2 times higher than seedlings treated with 0.75 and 1.25 kg a.i. ha^{-1} metazachlor, respectively, pointing towards an adverse effect of metazachlor on stem growth, irrespective of the dose applied (Table 3.1). Within three weeks after sowing 12.5% and 18.7% of the plants that were selected for the follow up of stem length, deceased in 0.75 and 1.25 kg a.i. ha^{-1} metazachlor treated plots, respectively (data not shown). Twenty-eight days after treatment, the initial inhibition of stem length development was still present. Remarkably, when considering aboveground biomass of plants at 28 DAT, fresh weight of plants in treated plots was two times higher than non-treated plants (Table 3.1). Qualitative 3,3´-diaminobenzidine (DAB) staining demonstrated that H_2O_2 concentrations were below the detection limit in leaves of nine-days-old plants irrespective of the condition. However, the occurrence of fasciation and atypical formation of the leaf edge in young leaves of metazachlor-exposed plants was present (Figure 3.1b). Changes in leaf

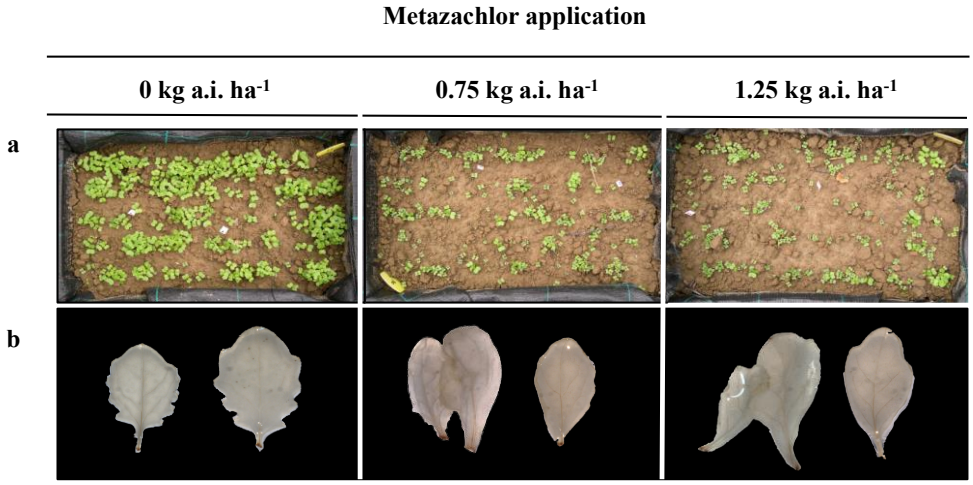

Figure 3.1. (a) *Overview of* B. napus *seedlings emerging in mesocosms, nine days after treatment (DAT) with 0, 0.75 and 1.25 kg metazachlor ha^{-1}. (b) At 19 DAT, H_2O_2 was determined in leaves using DAB staining. Leaves treated with 0.75 and 1.25 kg a.i. ha^{-1} metazachlor display changes in leaf morphology in form of straight leaf edges and fasciation in comparison with control leaves.*

morphology appeared in plants within one month after treatment with 0.75 and 1.25 kg a.i. ha^{-1} metazachlor and disappeared throughout their further development. *Brassica napus* treated with 1.25 kg a.i. ha^{-1} metazachlor contained less chlorophyll pigments than control plants at 19 DAT, which was mainly due to a decrease in chlorophyll *a* (Table 3.1). Together with this decline in chlorophyll concentration, the ratio of chlorophyll over carotenoids also decreased (Table 3.1). Whereas chlorophyll *a*, chlorophyll *b* and carotenoid concentrations remained unchanged over the following 10 days in non-treated plants, chlorophyll *a* concentrations increased significantly in time between 19 and 28 DAT in plants treated with metazachlor, resulting in a significantly higher chlorophyll content in the highest treatment condition.

3.4.1.2. *Long-term data*

The growth curve of *B. napus* demonstrates a faster increase of stem elongation of treated plants at the end of the winter (Supplementary file B). This accelerated stem elongation of treated plants coincided with an obvious acceleration in plant development (Supplemental file C). In early spring, the first

Table 3.1. *Short- and long-term parameters of* B. napus *treated with 0, 0.75 and 1.25 kg a.i. ha^{-1} metazachlor, when seeds were excessively sown. Data are shown as average values of minimal 5 biological replicates. * Significant decreases and increases are accentuated in light grey and dark grey, respectively, with post-hoc values: p < 0.05.*

Parameter	Unit	Metazachlor (kg a.i. ha^{-1}) 0	0.75	1.25
Short term				
Stem length - 9 DAT	cm	1.2 ± 0.14	**0.64 ± 0.04** *	**0.58 ± 0.04** *
Stem length - 28 DAT	cm	2.2 ± 0.2	**1.2 ± 0.1** *	**1.6 ± 0.2** *
Biomass - FW 28 DAT	g	0.6 ± 0.1	**1.1 ± 0.1** *	**1.3 ± 0.1** *
Pigments - 19 DAT				
Total Chlorophyll	µg/gFW	951 ± 24	876 ± 28	842 ± 16 *
Chlorophyll *a*	µg/gFW	801 ± 20	744 ± 23	720 ± 15 *
Chlorophyll *b*	µg/gFW	150 ± 7	132 ± 6	122 ± 3
Chlorophyll *a/b*		5.4 ± 0.2	5.7 ± 0.1	5.9 ± 0.2
Carotenoids	µg/gFW	213 ± 7	204 ± 5	203 ± 5
Chlorophyll/Carotenoid		4.5 ± 0.1	4.3 ± 0.1	4.2 ± 0.1 *
Pigments - 28 DAT				
Total Chlorophyll	µg/gFW	976 ± 23	1010 ± 19	1061 ± 22 *
Chlorophyll *a*	µg/gFW	813 ± 20	851 ± 17	890 ± 18 *
Chlorophyll *b*	µg/gFW	163 ± 4	160 ± 5	171 ± 5
Chlorophyll *a/b*		5.0 ± 0.1	5.4 ± 0.2	5.2 ± 0.1
Carotenoids	µg/gFW	213 ± 4.8	216 ± 4.6	225 ± 4.1
Chlorophyll/Carotenoid		4.59 ± 0.04	4.69 ± 0.05	4.72 ± 0.04
Long term				
Crop Density	plants/ mesocosm	93 ± 9.9	63 ± 7.2 *	56 ± 6.3 *
Plant biomass - dry weight	g/plant	0.54 ± 0.14	0.78 ± 0.08	0.94 ± 0.12 *
Seed weight	mg/seed	4.5 ± 0.1	5.2 ± 0.1 *	5.1 ± 0.2 *

flower buds appeared two weeks earlier in treated plants than in non-treated plants. In April, flowering of control plants was ten days later. The first flowers started to open in more than 90% of the plants at 219 DAT in 0.75 and 1.25 kg a.i. ha^{-1} treated plots and at 229 DAT in control plots. Early May, at 236 DAT, flowering was terminated in all plants in 0.75 and 1.25 kg a.i. ha^{-1} metazachlor treated plots, while 50% of the non-treated plants were still flowering. At the end of the growing season (June), seed weight and aboveground plant dry weight of plants treated with 0.75 and 1.25 kg a.i. ha^{-1} metazachlor was higher than for untreated plants (Table 3.1). Crop density was increasingly higher in control plots than in treated plots (Table 3.1), demonstrating that on the long-term, life history traits are more negatively influenced by initial competitive stress.

3.4.2. Mechanisms of herbicide-induced toxicity

3.4.2.1. *Short-term: Growth, development and morphology*

In order to exclude inter- and intraspecific competition, a modified experimental design was established whereby a fixed number of 36 seeds was sown. The absence of weeds in treated plots demonstrates the effectiveness of metazachlor as a herbicide (Table 3.2). The germination rate of *B. napus* at 7 DAT was 56-57% for all treatments. At this time point plants treated with 0.75 and 1.25 kg a.i. ha^{-1} metazachlor were smaller in size (Figure 3.2a). The reduction in individual plant size and crop density was still present at 28 DAT (Figure 3.2b). At this time point, the individual fresh weight of aboveground biomass of plants treated with 0.75 and 1.25 kg a.i. ha^{-1} metazachlor was 25 to 30% lower than that of non-treated plants, respectively (Table 3.2). Within one month after metazachlor application, several morphological symptoms of phytotoxicity were observed in the oilseed rape crop. Detailed photographs of leaves illustrate that metazachlor influenced morphological characteristics of *B. napus* leaves, inducing deformation of the leaf edge and fasciation of the first leaf pair at growth stage 12 (Figure 3.2c).

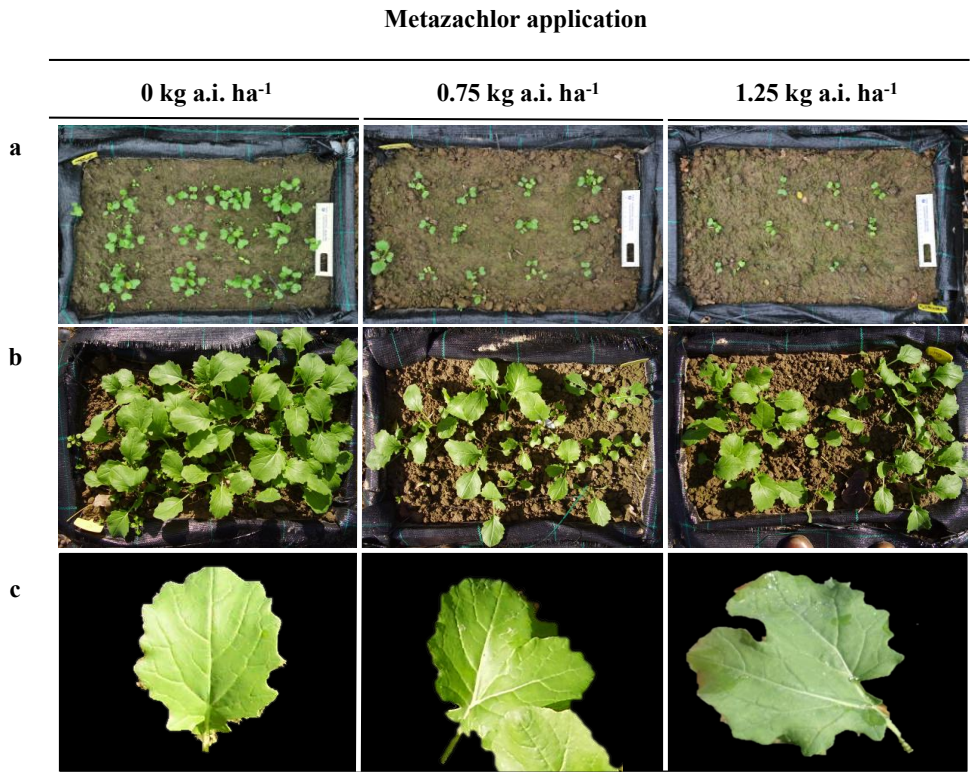

Figure 3.2. *Overview of mesocosms at 7 DAT (a) and 28 DAT (b) and detail of leaves (c) of* B. napus *seedlings at 28 DAT, after metazachlor treatment with 0, 0.75 and 1.25 kg metazachlor ha^{-1}.*

3.4.2.2. *Short-term: Lipid peroxidation, pigment and nutrient profiles*

Leaf tissues of treated plants did not contain higher levels of TBA-reactive metabolites than control plants, indicating that cell membranes remained intact under metazachlor treatment at 28 DAT (Table 3.2). The pigment profile of treated plants showed a change in composition as compared to controls. Whereas the carotenoid concentration was not affected by metazachlor treatment, chlorophyll *a* and *b* showed a diminishing trend (Table 3.2). However, this had no effect on the chlorophyll to carotenoid ratios. Small changes in nutrient profiles were observed in young leaves of *B. napus* (Table 3.2). Whereas plants from all conditions contained the same levels of macronutrients, the Na concentration was 40% and 30% higher in 0.75 and 1.25 kg a.i. ha^{-1} metazachlor-treated plants, respectively.

Table 3.2. *Short-term responses on growth, membrane integrity, pigment and nutrient profiles in* B. napus *leaf tissue, 28 days after treatment with 0, 0.75 and 1.25 kg a.i. ha^{-1} metazachlor. Data are shown as average values of minimal 5 biological replicates ± SE. * Significant decreases and increases are accentuated in light grey and dark grey, respectively, with post-hoc values: p < 0.05.*

Response level	Unit	Metazachlor (kg a.i. ha^{-1})		
		0	0.75	1.25
Efficiency of metazachlor				
Germination (7 DAT)	%	57 ± 3.4	56 ± 7.8	57 ± 5.6
Weed abundance (14 DAT)	%	100 ± 14	**4.7 ± 0.7** *	**3.4 ± 1.9** *
Growth				
Aboveground biomass	g	4.7 ± 0.45	3.7 ± 0.29	**3.3 ± 0.40** *
Membrane damage				
TBA reactive molecules	nmol/gFW	18 ± 1.2	20 ± 1.5	18 ± 1.8
Pigment profile				
Total Chlorophyll	µg/gFW	847 ± 48	806 ± 42	747 ± 27
Chlorophyll *a*	µg/gFW	688 ± 30	665 ± 27	623 ± 22
Chlorophyll *b*	µg/gFW	171 ± 19	127 ± 8	124 ± 8
Chlorophyll *a/b*		4.5 ± 0.2	4.9 ± 0.1	5.1 ± 0.3
Carotenoids	µg/gFW	176 ± 7	188 ± 7	171 ± 7
Chl/Car		4.6 ± 0.2	4.4 ± 0.04	4.4 ± 0.1
Nutrient profile				
Macronutrients				
K	mg/gDW	39.2 ± 2.1	37.2 ± 1.5	37.2 ± 0.6
Ca	mg/gDW	28.9 ± 0.5	28.5 ± 1.5	27.2 ± 1.1
S	mg/gDW	7.3 ± 0.3	7.7 ± 0.3	7.7 ± 0.4
P	mg/gDW	5.3 ± 0.1	5.7 ± 0.2	5.7 ± 0.5
Mg	mg/gDW	4.3 ± 0.3	4.1 ± 0.1	4.2 ± 0.1
Micronutrients				
Na	µg/gDW	498 ± 33	**701 ± 52** *	**651 ± 32** *
Fe	µg/gDW	146 ± 4	202 ± 46	191 ± 19
Zn	µg/gDW	40 ± 3	42 ± 3	49 ± 4
Mn	µg/gDW	23 ± 0	23 ± 1	23 ± 1
Cu	µg/gDW	3.2 ± 0.03	3.7 ± 0.2	3.4 ± 0.2

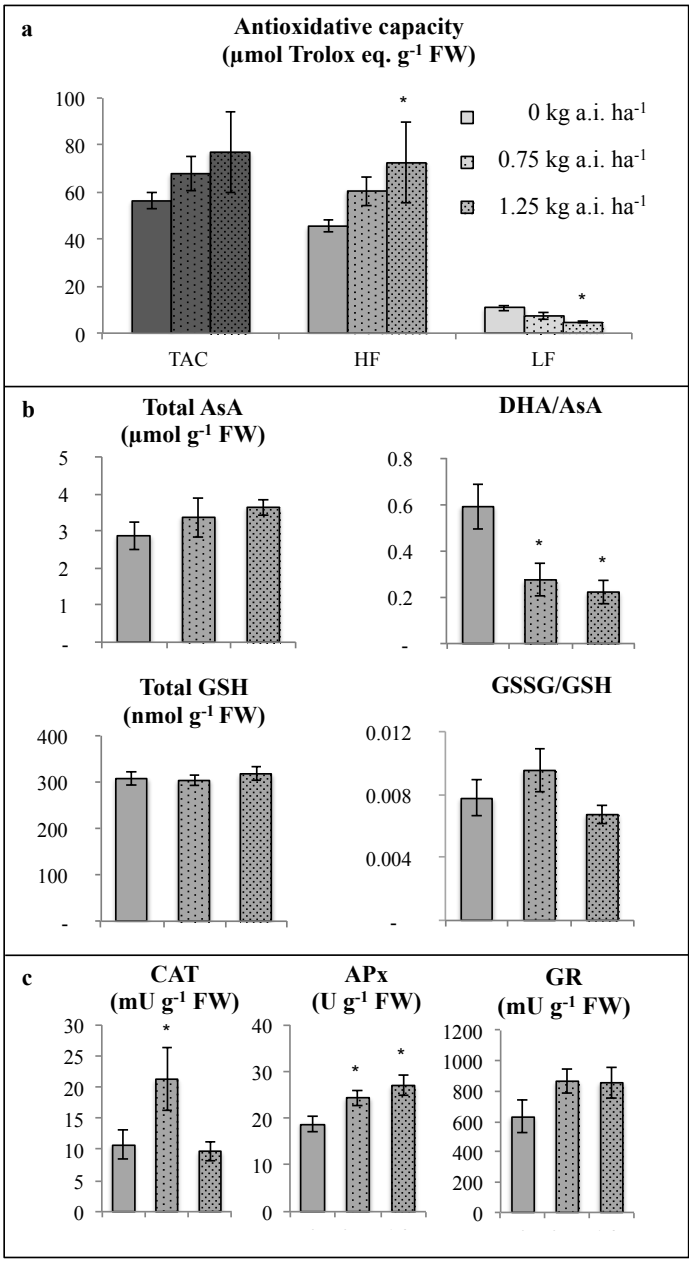

Figure 3.3. *(a) Total antioxidant capacity (TAC), hydrophilic (HF) and lipophilic fraction (LF) of antioxidants in* B. napus *leaf tissue, 28 days after treatment with 0, 0.75 and 1.25 kg a.i. ha^{-1} metazachlor. (b) Ascorbate (AsA) and glutathione (GSH) concentration and redox state and (c) antioxidant enzyme activities of catalase (CAT), asorbate peroxidase (APx) and glutathione reductase (GR) under similar conditions. Data are shown as average values of minimal 5 biological replicates ± SE. * post-hoc values: $p < 0.05$.*

3.4.2.3. *Short-term: Underlying processes of detoxification: antioxidant mechanisms and GST activities*

Assuming that the pre-emergent treatment of *B. napus* with metazachlor disturbs cellular redox processes, a closer look was taken at the presence of antioxidants (metabolites and enzymes). All parameters measured regarding detoxification and antioxidant capacity are represented in supplementary file D with the most significant results highlighted in Figure 3.3. Although the total antioxidative capacity of leaf cells did not differ between the different conditions, differences were observed in hydrophilic and lipophilic fractions of antioxidants in leaves at 28 DAT (Figure 3.3, Supplementary file D). The reducing capacity of the lipophilic fraction antioxidants decreased with increasing exposure to metazachlor. In contrast, the antioxidative capacity of the hydrophilic fraction increased with increasing metazachlor exposure. A more in depth examination of the hydrophilic fraction of antioxidant metabolites in *B. napus* leaves showed that the total concentration of ascorbate tended to increase with 18 to 27% in 0.75 and 1.25 kg a.i. ha^{-1} treated plants, respectively (Supplementary file D). Furthermore, the redox state of ascorbate shifted to the reduced form under the influence of metazachlor (Figure 3.3). Glutathione levels were situated around 304 to 318 nmol g^{-1} fresh weight in all conditions. No changes in glutathione redox state were found (Figure 3.3). Metazachlor treatment enhanced the activities of SOD, APx, GR, SPx and GPx with 20 to 40% (Supplementary file D), with significant increases in activity of CAT and APx (Figure 3.3). Glutathione S-transferases, which play an essential role in the conjugation of metazachlor metabolites to GSH, did not show any difference under metazachlor treatment for any of the tested substrates (Supplementary file D).

3.4.2.4. *Long term: Effect of chemical stress on yield of* Brassica napus *in the long term*

At harvest, stem height and fresh weight of aboveground biomass of *B. napus* were not influenced by metazachlor treatment. All measured life history traits, such as individual plant dry weight, seed yield per plant and seed weight were similar in control and metazachlor treated mesocosms (Table 3.3). On the long-term, nutrient profiles of the leaves were not influenced by herbicide treatment.

Concerning the characteristics of the seeds, the fatty acid profiles and the flavonoid profiles were similar for all treatments (Table 3.4). Concerning the nutrients in the seeds, an 8 to 15% increase of phosphorus was observed in plants exposed to 0.75 and 1.25 kg metazachlor ha^{-1}, respectively, and a 7% decrease of iron concentration after application of 1.25 kg metazachlor ha^{-1}.

Table 3.3. *Long-term yield and nutrient content of* B. napus*, after treatment with 0, 0.75 and 1.25 kg a.i. ha^{-1} metazachlor. Data are shown as average values of minimal 5 biological replicates ± SE. * Significant decreases and increases are accentuated in light grey and dark grey, respectively, with post-hoc values: $p < 0.05$.*

Response level	Unit	Metazachlor treatment (kg a.i. ha^{-1})		
		0	0.75	1.25
Biomass & yield				
Crop Density	plants/mesocosm	17 ± 2	15 ± 2	16 ± 2
Plant height	cm	76 ± 1	75 ± 2	73 ± 2
Plant biomass - DW	g/plant	7.8 ± 0.5	7.8 ± 1.4	7.0 ± 0.8
Seed yield	g/plant	2.3 ± 0.2	2.4 ± 0.5	2.3 ± 0.5
Seed weight	mg/seed	5.2 ± 0.1	5.2 ± 0.1	5.4 ± 0.1
Nutrient content aboveground biomass				
Macronutrients				
K	(mg/gDW)	17 ± 1	17 ± 1	17 ± 1
Ca	(mg/gDW)	20 ± 0.3	20 ± 1	21 ± 1
S	(mg/gDW)	5.4 ± 0.3	5.5 ± 0.2	5.6 ± 0.4
P	(mg/gDW)	2.0 ± 0.1	2.2 ± 0.1	2.2 ± 0.1
Mg	(mg/gDW)	1.6 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
Micronutrients				
Na	(µg/gDW)	123 ± 6	123 ± 7	120 ± 5
Fe	(µg/gDW)	69 ± 3	78 ± 11	63 ± 3
Zn	(µg/gDW)	17 ± 1	18 ± 1	16 ± 0.5
Mn	(µg/gDW)	6.5 ± 0.7	6.0 ± 0.6	5.6 ± 0.2
Cu	(µg/gDW)	1.18 ± 0.05	1.19 ± 0.04	1.15 ± 0.04

Table 3.4. *Oil quality parameters of* B. napus*, after treatment with 0, 0.75 and 1.25 kg a.i. ha^{-1} metazachlor. Data are shown as average values of minimal 3 biological replicates ± SE. * Significant decreases and increases are accentuated in light grey and dark grey, respectively, with post-hoc values: p < 0.05.*

Seed quality parameters	Unit	Metazachlor (kg a.i. ha^{-1})		
		0	0.75	1.25
Fatty acid profile				
C16:0	%	4.9 ± 0.0	4.8 ± 0.1	4.8 ± 0.1
C16:1	%	0.29 ± 0.01	0.27 ± 0.01	0.27 ± 0.02
C18:0	%	2.1 ± 0.0	2.0 ± 0.0	2.0 ± 0.0
C18:1	%	63 ± 0.4	63 ± 0.6	63 ± 0.8
C18:2	%	19 ± 0.3	19 ± 0.1	19 ± 0.4
C18:3	%	8.4 ± 0.1	8.5 ± 0.3	8.6 ± 0.3
C20:0	%	0.58 ± 0.01	0.58 ± 0.02	0.58 ± 0.01
C20:1	%	0.98 ± 0.01	0.99 ± 0.02	1.03 ± 0.03
C20:2	%	0.00 ± 0.00	**0.04 ± 0.02** *	**0.065 ± 0.004** *
C22:0	%	0.25 ± 0.00	0.25 ± 0.01	0.25 ± 0.01
C22 :1	%	0.131 ± 0.026	0.050 ± 0.025	0.083 ± 0.017
C22 :2	%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Flavonoid profile				
Kaempferol-glucoside	µmol/g	0.15 ± 0.01	0.16 ± 0.01	0.17 ± 0.01
Kaempferol-3-O-sinapoyl-sophoroside-7-glucoside	µmol/g	2.8 ± 0.2	2.7 ± 0.3	2.7 ± 0.4
Quercetin-3-O-phoroside	nmol/g	0.50 ± 0.04	0.46 ± 0.05	0.40 ± 0.07
Kaempferol-3-O-caffeoyl-sophoroside-7-O-glucoside	µmol/g	0.033 ± 0.004	0.026 ± 0.007	0.049 ± 0.010
Nutrient content in seeds				
Macronutrients				
K	(mg/gDW)	4.54 ± 0.03	4.55 ± 0.09	4.64 ± 0.04
Ca	(mg/gDW)	4.14 ± 0.04	4.23 ± 0.09	4.14 ± 0.04
S	(mg/gDW)	2.99 ± 0.04	2.94 ± 0.08	3.14 ± 0.05
P	(mg/gDW)	7.4 ± 0.1	**8.0 ± 0.2** *	**8.5 ± 0.1** *
Mg	(mg/gDW)	2.97 ± 0.04	3.09 ± 0.05	3.07 ± 0.05
Micronutrients				
Fe	(µg/gDW)	42 ± 0.9	40 ± 0.8	**39 ± 0.7** *
Zn	(µg/gDW)	27 ± 0.9	26 ± 0.4	26 ± 0.4
Mn	(mg/gDW)	33 ± 1.0	34 ± 0.9	32 ± 1.4
Cu	(µg/gDW)	2.2 ± 0.11	2.0 ± 0.02	2.0 ± 0.02
Na	(µg/gDW)	11 ± 0.2	11 ± 0.4	10 ± 0.3

3.5. Discussion

Since this study includes the investigation of the effects of short-term exposure of winter oilseed rape (*Brassica napus L.*) to the herbicide metazachlor and consequences towards its long-term yield, laboratory conditions could not meet the requirements for long-term testing. Therefore, a semi-controlled mesocosm experimental set-up was chosen to integrate full season examination (including winter). Terrestrial and aquatic mesocosm experiments have been considered valuable test systems for hazardous chemicals (Crossland and La Point 1992, Morgan and Knacker 1994).

The absence of weeds in treated plots confirmed the efficiency of metazachlor as a herbicide (Dixon and Clay 2004). However, as noticed in earlier studies the effects on the non-targeted crop should not be underestimated (Mohr *et al.* 2008, Baćmaga *et al.* 2014). Therefore growth and development of *B. napus* were followed during the entire growing season, whereby seeds were sown either abundantly or controlled and metazachlor was applied in two concentrations, as recommended (0.75 kg a.i. ha^{-1}) and as often applied in practice (1.25 kg a.i. ha^{-1}) (Fytoweb). When seeds were sown in excess, germination of *B. napus* was adversely affected by pre-emergent application of metazachlor. Metazachlor application resulted in a reduction of seed emergence and a reduction of survival of young crop seedlings shortly after emergence, leading to a decreased crop density in treated plots within two weeks after treatment (Figure 3.1a). At emergence (9 DAT) stem growth of metazachlor-treated seedlings was inhibited. The reduced stem height was still present at 28 DAT. Willoughby *et al.* (2006) have pointed out similar reductions of stem height in tree species due to metazachlor treatment. However, when taken into account the fresh weight biomass of one-month-old seedlings, metazachlor-treated plants demonstrated a clearly higher fresh weight than control plants. This observation could be the result of the higher crop density present in control mesocosms. The high density of plants caused competition for capturing light between the individual plants, which resulted in increased investing in height over biomass. The reduction of fresh weight of non-treated *B. napus* seedlings points out, together with the stagnation of chlorophyll concentration under this

treatment, that intraspecific competition outcompetes chemical stress induced by metazachlor. In order to exclude this initial intraspecific competition effect, plant density in the control mesocosms was reduced to an equal number as in the metazachlor-treated mesocosms. The development (*i.e.* stem elongation, inflorescence emergence and flowering) of metazachlor-treated plants resumed earlier in spring, resulting in the production of more heavy seeds at the end of the season. These results suggest that the faster development is most likely linked with the increased availability of space and nutrients for each crop plant in treated plots, due to the initial absence of intraspecific competition on the one hand and on the other hand the relatively short half-life of metazachlor in soil, which comprises three to four months (Mamy *et al.* 2005, Rouchaud *et al.* 1992). In a field trial it was found that metazachlor was completely metabolised within 88 days in *B. napus* (Koleva L., personal communication).

When seeds were either sown in excess or in a limited amount, several symptoms of metazachlor-induced toxicity were observed in young *B. napus* seedlings within one month after herbicide application, at both morphological and physiological level. Regardless of the amount of seeds sown, seedlings were less abundant and smaller in metazachlor-treated plots, indicating that germination was inhibited and growth was retarded. It has been illustrated before that alachlor and metolachlor, two related chloroacetamide herbicides, inhibit cell elongation and cell division, resulting in distorted seedling settlement and growth, which might explain our observation (Deal and Hess 1980). A minor number of plants also displayed deviating leaf growth in their early growth stages (Figure 3.1b). The occurrence of fasciation is connected with the application of metazachlor, since this morphological deformation of the leaves was not observed in leaves of unexposed crops. This can be a direct result of the selective working mechanism of metazachlor, whereby membrane formation is impaired and lipid biosynthesis is altered (Böger 2003, Deal and Hess 1980). The manifestation of leaf deformation and fasciation due to chloroacetamide herbicide treatment, has been reported before for propachlor (Dhillon and Lamar 1972), but has not yet been reported for metazachlor. Fasciation of leaves was transient and most likely related to the short half-life of metazachlor in the soil (Mamy *et al.* 2005).

At the cellular level, the total chlorophyll concentration in leaves of *B. napus* was lower shortly after exposure to metazachlor, regardless of the amount of seeds that were sown (Table 3.1 and 3.2). These results suggest that either pigment biosynthesis is inhibited or that present pigments are degraded under influence of the active ingredient metazachlor. Reduced pigment concentrations in crops under influence of herbicides from multiple classes have been reported before (Yuan *et al.* 2013). Based on the working mechanism of metazachlor, whereby lipid biosynthesis is inhibited, it is most probable that the biosynthesis of pigments is disturbed.

When intraspecific competition stress was excluded by optimizing crop density (around 48-60 plants per m^2), symptoms of metazachlor-induced phytotoxicity became more apparent. Stem height, being a sensitive biometric parameter for metazachlor-induced stress in *B. napus,* was strongly suppressed under metazachlor treatment. Moreover, metazachlor resulted in a clear reduction of seedling fresh weight at 28 DAT. Simultaneously, metazachlor induced apparent effects at cellular level. Although no signs of lipid peroxidation of the cell membrane were observed at 28 DAT, the lipophilic fraction of antioxidants, such as tocopherols and carotenoids, decreased under metazachlor exposure, which may be due to direct inhibition of lipid biosynthesis by metazachlor (Böger 2003). The detoxification capacity of *B. napus* leaf cells did not seem to be influenced by metazachlor. GST activities, tested for different substrates, were similar in the different conditions 28 DAT, suggesting that direct detoxification of metazachlor was transient, which should be verified by measurements in a short-term kinetic setup.

Although little or no adverse effects were detected at cell membrane structure, pigment and nutrient profiles, changes in the antioxidative defence processes were observed. The activity of antioxidative enzymes increased together with the antioxidative capacity of hydrophilic metabolites (Figure 3.2). More specifically, the increased activity of APx and the shift in ascorbate redox state towards the reduced state, indicate the activation of the AsA-GSH cycle and consequently the presence of ROS in leaf cells. These observations suggest that detoxification of metazachlor is not sufficient in the prevention of ROS

generation at cellular level. However, the antioxidant defence mechanisms seem to provide sufficient capacity to prevent structural damage to the leaf cells.

The long-term yield and oil quality traits, such as biomass, seed production and seed weight, nutrient content, flavonoid and fatty acid profiles of the oil were not affected by pre-emergent metazachlor exposure. However, it is noteworthy to mention that not all agricultural practices were included in this study. It is common use in agricultural practices that metazachlor is applied for a second time in the early spring to ensure weed growth inhibition. This second application could have implications for biomass production and oil quality parameters too. Furthermore, it is worth to mention that the effects of metazachlor on summer oilseed rape should also be investigated. The shorter growing period of this variety and the absence of a growth stop during winter, which allows winter oilseed rape to recover, can be disadvantageous in the sense of metazachlor toxicity.

3.6. Conclusions

This study shows that metazachlor application has a direct negative influence on *B. napus* seedling survival and growth in the short-term. Considering the retarded emergence and growth, morphological leaf deviations and decreased chlorophyll content, it can be concluded that initially, shortly after exposure, the non-target crop *B. napus* displays metazachlor-induced symptoms of phytotoxicity. The increase in hydrophilic fraction of antioxidant metabolites, such as ascorbate, together with the increase in antioxidant enzyme activities suggest an important role of the antioxidant defence mechanism in the cellular protection against herbicide-induced ROS. Nevertheless, the pre-emergent application of metazachlor did not impose negative effects on growth and development of *B. napus* in the long-term, leading to an identical yield and quality of its long-term end products at harvest. Therefore, it can be stated that the initial phytotoxic responses of *B. napus* are transient in time, which could be explained by a rapid turnover of metazachlor in the plant and the half-life time of metazachlor in the soil.

3.7. References

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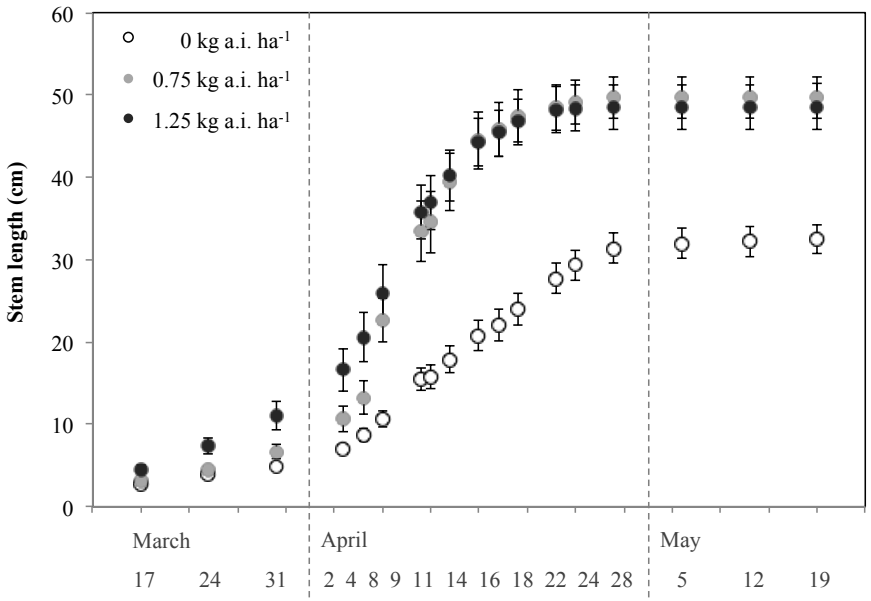
3.8. Supplementary files

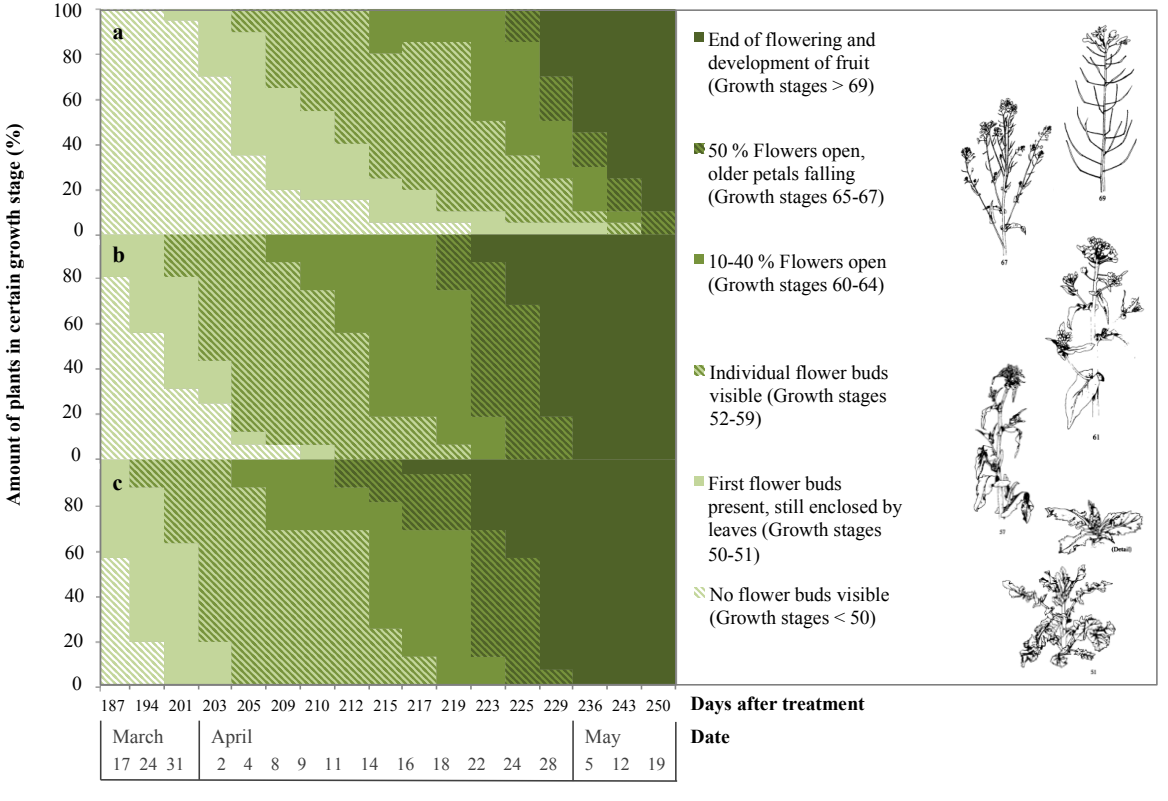
Supplementary file A. *Experimental design and soil properties of soil collected from farmland at Alken in two successive growing seasons (September 2012 to June 2013 and September 2013 to June 2014).*

Experiment	1. Excess Sowing	2. Controlled sowing
Experimental design		
Amount of seeds sown per mesocosm	In excess (>100)	36
Application of Metazachlor	Pre-emergent	Pre-emergent
Soil Characteristics		
Soil type	Loam	Loam
pH	5.7	6.1
C	1.02	0.78
Plant available nutrients		
P (mg/kg soil))	230	250
K (mg/kg soil)	200	240
Mg (mg/kg soil)	110	160
Ca (g/kg soil)	1.06	1.35
Na (mg/kg soil)	< 0.9	1.7
N (mg/kg soil)	n.d.	125

Supplementary file B. *Growth curve of* B. napus *after winter stop, based on stem length (cm) measurements, treated with 0 (o), 0.75 (•) and 1.25 (•) kg metazachlor ha^{-1}. Data are represented as average value of 16 biological replicates ± SE.*



Supplementary file C. *Inflorescence and plant flowering of* B. napus *in March, April and May, treated with 0 (a), 0.75 (b) and 1.25 (c) kg metazachlor ha^{-1}, expressed as the amount of all measured plants (%) that are situated in a specific growth stage at a certain timepoint. For each condition, 16 biological replicates were used. Different growth stages are depicted as described by Lancashire* et al. *(1991).*



Supplementary file D. *Short-term responses on antioxidant defence and detoxification in* B. napus *leaf tissue, 28 days after treatment with 0, 0.75 and 1.25 kg metazachlor ha^{-1}. Data are shown as average values of minimal 5 biological replicates ± SE. * Significant decreases and increases are accentuated in light grey and dark grey, respectively, with post-hoc values: p < 0.05.*

Response level	Unit	Metazachlor (kg a.i. ha^{-1})		
		0	0.75	1.25
Antioxidant defence and detoxification				
Total antioxidative capacity	µmol Trolox eq./gFW	56 ± 3	68 ± 7	77 ± 17
Hydrophilic fraction AO	µmol Trolox eq./gFW	46 ± 2	60 ± 6	**72 ± 17** *
Lipophilic fraction AO	µmol Trolox eq./gFW	10.7 ± 1.2	7.4 ± 1.5	**4.5 ± 0.4** *
Antioxidative enzymes				
SOD	mU/g FW	398 ± 60	528 ± 76	515 ± 60
CAT	mU/g FW	11 ± 2	**21 ± 5** *	10 ± 2
APx	U/g FW	19 ± 2	**24 ± 2** *	**27 ± 2** *
GR	mU/g FW	634 ± 111	865 ± 78	855 ± 100
SPx	mU/g FW	69 ± 20	89 ± 13	63 ± 13
GPx	mU/g FW	53 ± 9	106 ± 20	74 ± 17
Antioxidative metabolites				
Total ascorbate	µmol/gFW	2.9 ± 0.4	3.4 ± 0.5	3.6 ± 0.2
Redox state ascorbate	DHA/ASA ratio	0.59 ± 0.10	**0.27 ± 0.07** *	**0.22 ± 0.05** *
Glutathione	nmol/gFW	308 ± 14	304 ± 12	318 ± 15
Redox state glutathione	GSSG/GSH ratio	0.008 ± 0.001	0.010 ± 0.001	0.007 ± 0.001
Detoxifying enzymes				
GST-CDNB	mU/g FW	208 ± 12	229 ± 17	226 ± 12
GST-Fluorodifen	mU/g FW	5.0 ± 0.8	4.3 ± 0.7	6.7 ± 1.8
GST-Npa	mU/g FW	36 ± 4	45 ± 4	38 ± 4

Chapter 4 Short-term responses of *Brassica napus* to pre-emergently applied metazachlor: a microcosm study

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4.1. Abstract

In accordance with realistic application approaches, a short-term one-factorial experiment was set up to investigate the phytotoxic impact of pre-emergent application of the chloroacetamide herbicide metazachlor on *Brassica napus*. In addition to morphological parameters, the underlying processes that ultimately determine the extent of herbicide-induced phytotoxicity, *i.e.* herbicide metabolisation and cellular antioxidant defence, were examined. The present study demonstrated that metazachlor provoked fasciation of the leaves closely after emergence, which could possibly be addressed to its working mechanism whereby cell division is impaired through the inhibition of very long chain fatty acid synthesis. The increased activities of antioxidative enzymes and metabolites in leaf tissue indicated the presence of reactive oxygen species under the influence of metazachlor. This resulted in oxidative damage in the form of membrane lipid peroxidation. Simultaneously, the increased activity of glutathione S-transferase (GST) and the shift in glutathione (GSH) redox state suggested the activation of the detoxification metabolism. This occurred however at the expense of growth, with a temporary reduction in plant height and weight after application. The results suggested that the internal metazachlor concentration had disappeared within 3 to 4 months after application, which resulted in the recovery of the crop. In conclusion, metazachlor induces phytotoxicity in the short-term, either directly through its mode of action or indirectly through the induction of oxidative stress, which resulted in a temporary reduction in growth.

4.2. Introduction

Metazachlor is a pre-emergently used herbicide in the cultivation of the oilseed crop, *Brassica napus*, to chemically prevent the settling and growth of broadleaved weeds and annual grasses. As a chloroacetamide, metazachlor is known for inhibiting lipid biosynthesis and hence the formation of very long chain fatty acids (VLCFA) (Fuerst 1987). Like most herbicides, metazachlor can affect other non-target species via soil infiltration, drainage and run-off (Mamy *et al.* 2005, White and Boutin 2007, Carpenter and Boutin 2010). Even before it enters the ecosystem, the herbicidal compound can potentially affect the cultivated crop. This can subsequently result in reductions in yield (Foy and Witt

1990, Felix *et al*. 2007), morphological aberrations (DeRidder and Coldsbrough 2006), induction of oxidative stress (Cui *et al*. 2010), increased lipid peroxidation of membranes (Jiang *et al*. 2010) and decreased chlorophyll content (Le Yin *et al*. 2008). Results derived from field experiments often display large variations between plots from a certain condition, resulting in a lack of statistical support. This is due to the fact that field experiments cannot exclude the complex interaction of external factors, such as direction and rate of drainage, the presence of herbivores (*e.g.* snails), heterogeneity of the soil, *etc*. This microcosm study aims to exclude these side effects by the use of a one-factorial experimental set-up and is therefore complementary to the mesocosm experiment previously conducted (Chapter 3).

The occurrence and the extent of phytotoxicity of metazachlor in *B. napus* are determined either by the crop's capacity to detoxify the herbicide on one hand and its capacity to cope with metazachlor-induced oxidative stress on the other hand. The detoxification metabolism of a crop plays an important role in the tolerance of a crop against herbicides, with a significant role for glutathione S-transferase (GST) (Jablonkai and Hatzios 1993, Hatton *et al.* 1996). Once herbicidal compounds are present in the cell cytoplasm, the structure and reactivity of the compound will be modified by cytochrome P450 proteins in the first phase of detoxification. In the second phase, the modified herbicidal compound is conjugated with glutathione (GSH) by the action of GSTs. In the third phase of herbicide detoxification, GSH will function as a tag for the compartmentalization of the herbicidal compound into either the vacuole or the cell membrane. Herbicidal compounds are known to induce the activity of GST in most crops species (Cole 1994, Scarponi *et al.* 2006, Cui *et al.* 2010). Based on their sequence identity, gene organisation and active site residues, plant GST's can be divided into five classes: tau, phi, theta, zeta and lambda (Dixon and Edwards 2010). Tau and phi class GSTs are plant-specific and do not occur in mammalian species like all other plant GST classes. GST isoenzymes that belong to the same class have a 40 to 60% identity in their primary structure. Structurally, GSTs are composed of two subunits that can be either identical (homo-dimeric) or distinct (hetero-dimeric). Each subunit contains a kinetically independent active site with distinct domains for the GSH (G-site) and the

electrophilic substrate (H-site). With each subunit encoded by a separate gene, plants contain complex multigene families of GSTs. Hence, the various subunits may be able to dimerise in many permutations, producing multiple homo- and hetero-dimeric GST isoenzymes (Dixon *et al*. 1998). GST isoenzymes involved in xenobiotic metabolism are subjected to discrete regulation, showing distinct but overlapping substrate specificities. From complementation studies, it is likely that quite dissimilar GSTs share similar functions (Edwards 2000). Despite the association between GSTs and plant stress responses, it remains unclear whether different GST classes are substrate specific. The inducibility of phi and tau class GSTs after plant exposure to either biotic or abiotic stresses is a characteristic feature of these genes (Marrs 1996). Several tau class GSTs are known to be strongly induced during cell division (Marrs 1996). Phi class GSTs have been shown to be highly reactive towards chloroacetamide and thiocarbamate herbicides (Cho *et al*. 2006, Cole 1994).

Initially after herbicide application, when detoxification is either activated or at its full turnover, the non-detoxified fraction of cytosolic herbicidal compounds can indirectly cause oxidative damage at different cellular levels by the induction of oxidative stress. Herbicide-induced oxidative stress has been described in crops (Gill and Tuteja 2010, Faheed 2011) and for chloroacetamides, such as alachlor and metolachlor, in particular (Štajner *et al.* 2001, Liu *et al.* 2012). Herbicides from different classes of mode of action can negatively affect crop morphology (Dean *et al.* 1990) and physiology, ranging from destabilisation of cellular membranes (Dayan and Watson 2011) to pigment profiles (Kopsell *et al.* 2011). However, the underlying mechanisms of phytotoxicity are not well addressed in literature. Due to the relatively short degradation rate of metazachlor, which ranges between three to four months, it is important to monitor metazachlor-induced phytotoxicity closely after application in this short-term microcosm study. A one-factorial microcosm experiment was set up (i) to monitor growth, development and herbicide uptake of the crop *B. napus* during 9 weeks after treatment with metazachlor and (ii) to monitor cellular structure, such as membrane integrity, pigment and nutrient content, and cell functioning, such as herbicide detoxification and antioxidant defence mechanism, of *B. napus* within 2 and 4 weeks after pre-emergent application of metazachlor.

4.3. Experimental design and methodology

Three days before sowing, *B. napus* (cultivar *Remy*) seeds were surface sterilised. Hereby, seeds were washed in a 0.1% sodium hypochlorite solution for 2 min and subsequently rinsed thoroughly with deionised water. Next, seeds were rinsed in deionised water during 20 min and then stored in a closed Petri dish on a moistened filter. After being incubated in the dark at 4 °C during two nights, seeds were separately sown in microcosms on 1.3 kg sandy soil at 1 cm depth. In each microcosm six seeds were sown. The day after the seeds were sown, 10 mL of metazachlor solution was applied on the soil surface in the following concentrations: 0 mM, 0.2 mM and 0.4 mM metazachlor, which corresponded with 0 mg, 0.5 mg and 1 mg active ingredient per microcosm. Every two days, 10 to 50 mL ½ Hoagland nutrient solution was applied. Plants were grown in a growth chamber under controlled environmental conditions set at a 12 h photoperiod, 65% relative humidity and day/night temperatures of 22 °C and 18 °C, respectively. A combination of blue, red and far-red LED modules simulated the photosynthetic active radiation (PAR) of 200 µmol $m^{-2}s^{-1}$ of sunlight. Germination was determined by counting the percentage of seeds emerged within seven days after metazachlor application. Thereafter, the amount of plants was reduced to one plant per microcosm. Growth was monitored daily during 7 weeks by determining the growth stage of each individual plant according to Lancashire *et al.* (1991). Metazachlor uptake into the aboveground plant parts was monitored 14, 28 and 42 days after treatment (DAT). Leaf tissue for biochemical analyses was collected 14 and 28 DAT, snap frozen in liquid nitrogen and subsequently stored at -70 °C. During sampling, weight, root and shoot length were measured. All biochemical analyses were conducted as described in Chapter 2. In addition to these time points, fresh weight was recorded at 9 weeks after treatment (63 DAT).

4.4. Results

4.4.1. Growth, development and morphology of *B. napus*

Seven days after metazachlor application, seed germination was 10% lower in microcosms treated with 0.2 and 0.4 mM metazachlor in comparison to the non-treated microcosms, although not significant (Figure 4.1A). Two weeks after treatment (14 DAT), 1.12 and 1.58 mg metazachlor/kg FW were found in the aerial parts of 0.2 and 0.4 mM metazachlor-treated plants, respectively. During the following four weeks, the levels of metazachlor decreased with 78% and 64% in the aerial plant parts of the respective treatments to 0.24 and 0.57 mg metazachlor/kg FW at 42 DAT (Figure 4.1B).

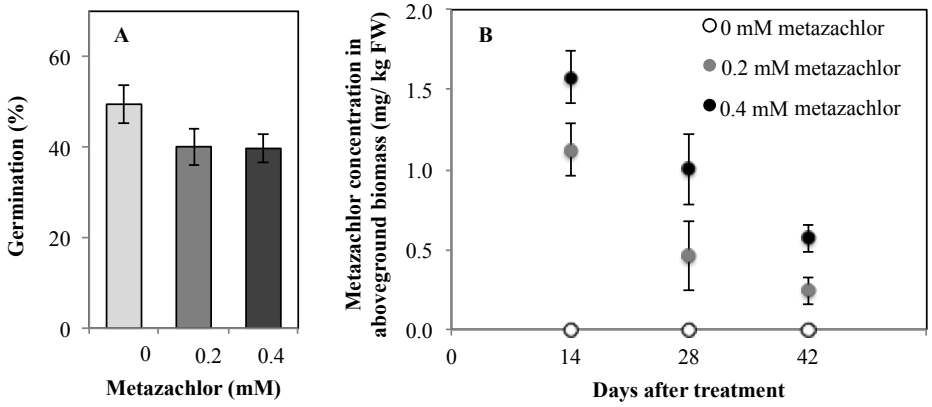

Figure 4.1. *(A) Percentage of* B. napus *seedlings that germinated within 7 DAT with 0, 0.2 and 0.4 mM metazachlor. Data are presented as average values of minimal 18 biological replicates ± SE. (B) Metazachlor concentration in aboveground plant parts of* B. napus *14, 28 and 42 DAT with 0 mM, 0.2 mM and 0.4 mM metazachlor. Data are presented as average values of minimal 3 biological replicates ± SE.*

Closely after application leaves displayed fasciation in the form of crinkled leaves, shortened mid ribs and incomplete detachment of leaves under the influence of metazachlor (Figure 4.2A). These malformations appeared within the first two to six weeks after treatment and remained present until differences still leaves' abscission during further development. Twenty-one DAT 20% and 50% of the plants treated with 0.2 and 0.4 mM metazachlor, respectively, displayed signs of fasciation and 20% of the plants in both treatments did not survive the first seven weeks (data not shown). Weight and height were strongly

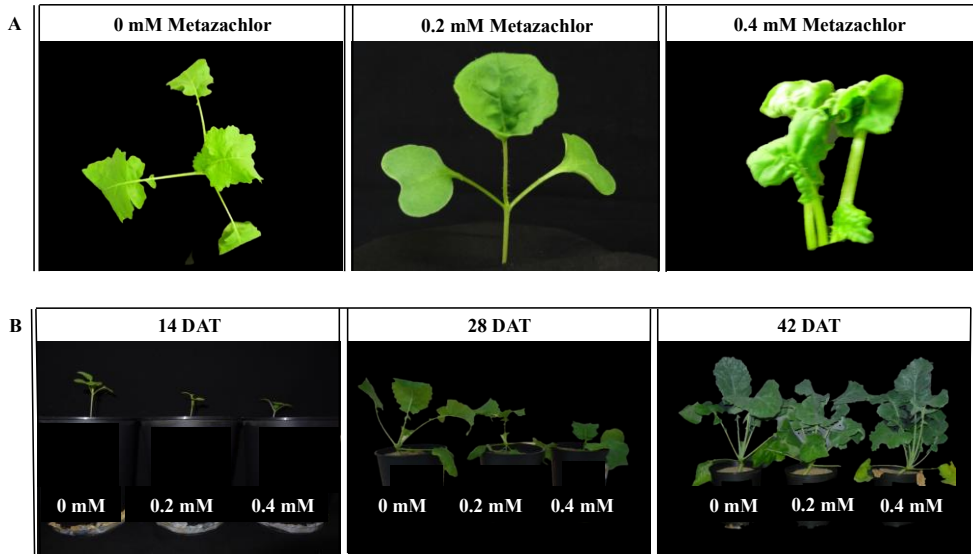

Figure 4.2. *(A) Detailed images of* B. napus *exposed to 0 mM, 0.2 mM and 0.4 mM metazachlor at 21 DAT. Plants exposed to 0.2 mM and 0.4 mM metazachlor displayed malformations of the leaves. (B) Overview images of* B. napus *exposed to 0 mM, 0.2 mM and 0.4 mM metazachlor at 14, 28 and 42 DAT.*

inhibited in 0.2 and 0.4 mM metazachlor-exposed plants at 14 DAT in a dose dependent way (Figures 4.2B, 4.3A and Table 4.1). Twenty-eight DAT, these differences still existed (Figure 4.3B and Table 4.1), but at 63 DAT fresh weights of plants under different exposures were equal (Figure 4.3C). When taking a

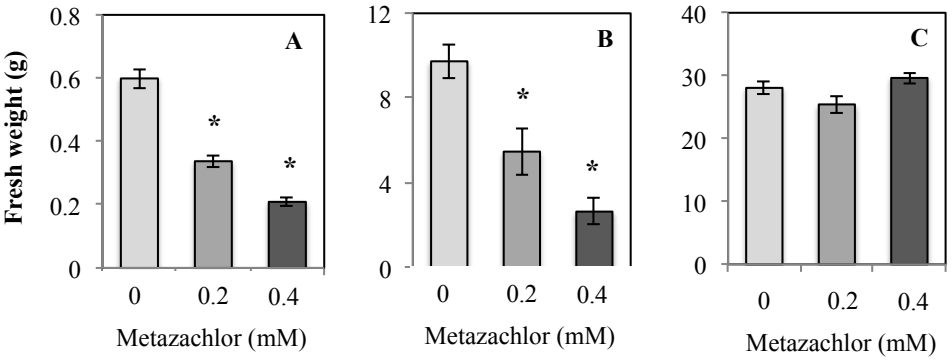

Figure 4.3. *Aboveground fresh weight of* B. napus *(A) 14 DAT, (B) 28 DAT and (C) 63 DAT with 0 mM, 0.2 mM and 0.4 mM metazachlor. Data are presented as average values of minimal 10 biological replicates ± SE. (* post-hoc values: $p < 0.05$)*

closer look into the rate of development of leaves of the young seedlings, no apparent differences were observed up to five weeks after metazachlor application (35 DAT) (Figure 4.4). However, considering leaf surface area and petiole length, the leaves appeared to be smaller under the influence of metazachlor, with smaller leaf surface area and shorter petioles which might explain the reduction in aboveground weight in the short term (Supplementary file A). Between five and seven weeks after application, metazachlor-exposed plants tended to develop leaves faster than control plants. At 47 DAT, for example, 30% and 70% of 0.2 and 0.4 mM metazachlor-exposed plantswere situated in growth stage 19 or 20, respectively, meaning that these plants had developed 9 or more leaves whereas all control plants had developed maximal 8 leaves (Figure 4.3).

4.4.2. Enzymes and metabolites involved in detoxification

The activity of GST significantly increased under the influence of metazachlor, at 14 and 28 DAT (Table 4.1, Figure 4.5). The activity of GST was increased towards chlorodinitrobenzene (CDNB), fluorodifen and nitrophenylacetate (Npa) substrates and not found for 1,2-dichloro-4-nitrobenzene (DCNB), 4-nitrobenzyl chloride (NBC) and p-nitrobenzpyl chloride (NBoC), with the strongest induction towards CDNB (Table 4.1). At both time points, the level of GSH, which is consumed during GST-catalysed metabolisation of xenobiotic compounds such as herbicides, tended to be lower under the influence of metazachlor (Figure 4.4). Whereas this trend could not be statistically underpinned, the redox state of glutathione was significantly turned towards the oxidised form (GSSG) at 14 DAT, under influence of 0.4 mM metazachlor (Table 4.1). At 28 DAT, the redox state of GSH was similar in all treatments (Table 4.1).

4.4.3. Metazachlor induces oxidative stress in the leaves of *B. napus*

The presence of reactive oxygen species (ROS), *e.g.* H_2O_2, was visualised using 3,3'-diaminobenzidine staining. No differences in the presence of H_2O_2 were detected at 14 DAT since DAB staining was restricted to the veins in all conditions (Supplementary file B). An increasing trend in total antioxidative capacity (TAC) was observed in leaf cells under the influence of metazachlor at

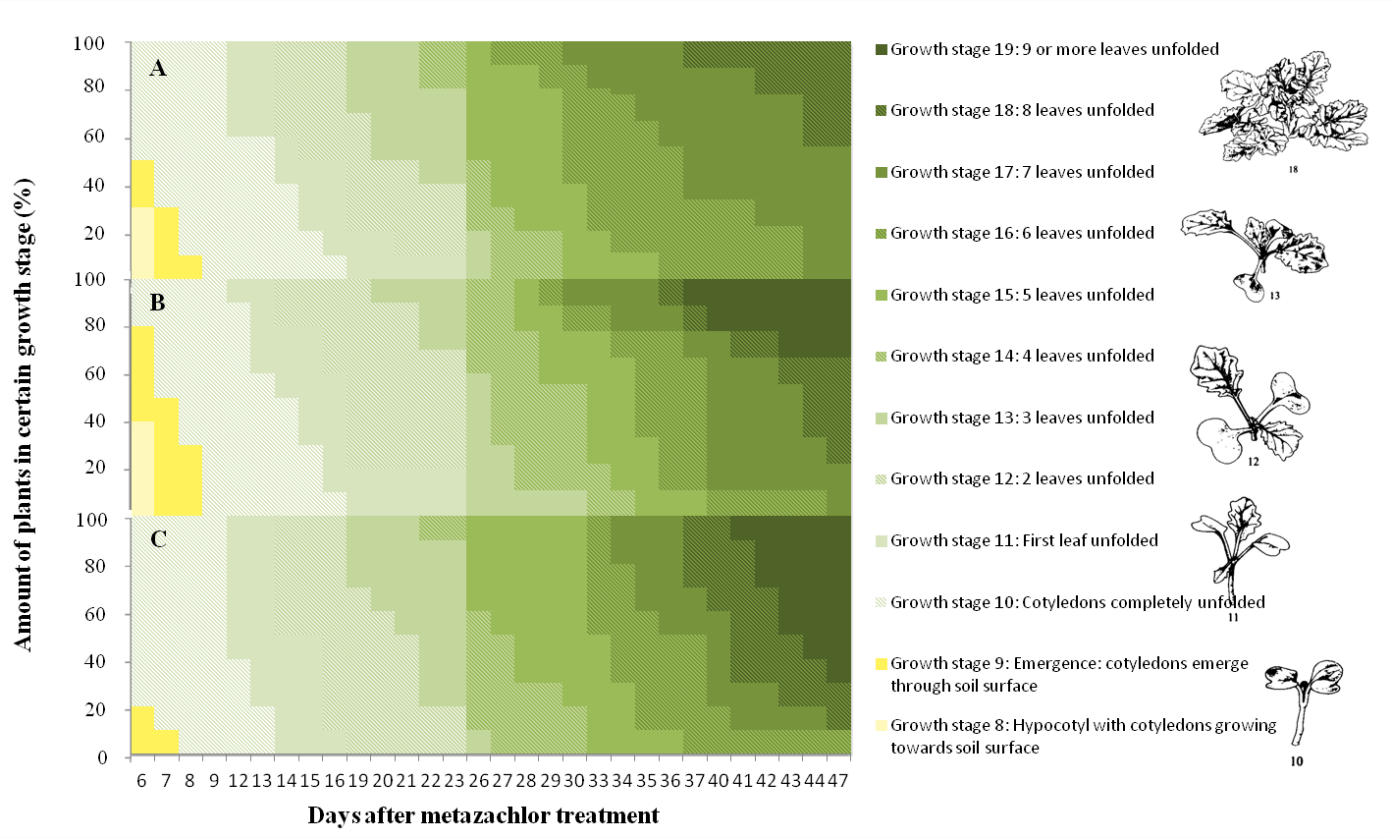

Figure 4.4. *Emergence (yellow) and leaf development (green) of* B. napus *over time, treated with (a) 0 mM, (b) 0.2 mM and (c) 0.4 mM metazachlor, expressed as the amount (%) of all measured plants that are situated in a specific developmental stage at a certain time point (DAT). Different growth stages are depicted as described by Lancashire et al. 1991.*

both 14 and 28 DAT (Table 4.1). At 14 DAT, the total antioxidative capacity (TAC) in the leaves of *B. napus* tended to increase under the influence of metazachlor, however this trend could not be statistically supported (Table 4.1). At 28 DAT, a significant increase in the lipophilic fraction of antioxidants was observed (Table 4.1). Additionally, the activities of enzymes involved in the antioxidative defence (SOD, CAT, APx and GR) and cell wall lignification (GPx and SPx) were measured. In general, the activities of all antioxidative enzymes increased with exposure to increasing metazachlor doses (Table 4.1). A higher activity in metazachlor-treated plants in comparison to non-treated plants could be statistically confirmed for catalase (CAT), ascorbate peroxidase (APx) and syringaldazine peroxidase (SPx) at 14 DAT and for superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and guaiacol peroxidase (GPx) at 28 DAT. Twenty-eight DAT, the levels of AsA were not affected by the applied metazachlor treatments. Together with AsA, GSH is a key metabolite in the AsA-GSH cycle, a supportive cycle behind the enzymatic antioxidative defence. The redox state of GSH was significantly leaning towards the oxidised form (GSSG) under the influence of 0.4 mM metazachlor at 14 DAT.

4.4.4. Metazachlor induces membrane lipid peroxidation and shifts in pigment and nutrient profiles

The increased presence of TBA-reactive metabolites of 39% and 43% in 0.2 mM and 0.4 mM metazachlor-exposed plants, respectively, indicated that cellular membranes were destabilised by lipid peroxidation at 28 DAT (Table 4.1). This observation was supported by a 67% increment of potassium leakage in 0.4 mM metazachlor-exposed plants (Table 4.1). No clear shifts in pigment profile were observed two weeks after application. Twenty-eight DAT however, the pigment profile in *B. napus* leaves was influenced by 0.2 mM metazachlor resulting an increased chlorophyll concentration, due to an increment of chlorophyll *a* (Table 4.1).

Table 4.1. (pages 104 and 105) *Parameters concerning growth, membrane damage, pigment and nutrient profile, antioxidative defence and detoxification in* B. napus *leaves, 14 and 28 DAT with 0 mM, 0.2 mM and 0.4 mM metazachlor. Data are shown as average values of minimal 4 biological replicates ± SE. * Significant decreases and increases are accentuated in light grey and dark grey, respectively, with post-hoc values: $p < 0.05$.*

Response level	Unit	14 Days after metazachlor treatment Metazachlor treatment 0 mM	0.2 mM	0.4 mM
Growth				
Shoot weight	mg	599 ± 31	**338 ± 19**	**209 ± 15**
Root weight	mg	119 ± 9	**52 ± 4**	**28 ± 2**
Shoot length	cm	4.4 ± 0.3	**2.4 ± 0.1**	**1.7 ± 0.2**
Root length	cm	12.0 ± 0.5	**6.0 ± 0.5**	**3.1 ± 0.3**
Antioxidant defence and detoxification				
Total antioxidative capacity	µmol Trolox eq./gFW	18 ± 2	23 ± 1	23 ± 2
Hydrophilic fraction AO	µmol Trolox eq./gFW	15 ± 2	20 ± 1	20 ± 1
Lipophilic fraction AO	µmol Trolox eq./gFW	2.8 ± 0.3	3.3 ± 0.3	3.9 ± 0.8
Antioxidative enzymes				
SOD	mU/g FW	293 ± 32	379 ± 45	370 ± 73
CAT	mU/g FW	7.5 ± 1.4	**13.5 ± 1.8**	9.4 ± 1.4
APx	U/g FW	38 ± 3.4	**54 ± 0.9**	52 ± 9.8
GR	mU/g FW	470 ± 48	671 ± 97	863 ± 140
SPx	mU/g FW	39 ± 6	**178 ± 74**	**241 ± 38**
GPx	mU/g FW	47 ± 3.4	127 ± 37	109 ± 36
Antioxidative metabolites				
Total ascorbate	µmol/gFW	4.3 ± 0.7	4.3 ± 0.5	5.7 ± 0.6
Oxidised ascorbate	DHA/ASA ratio	0.19 ± 0.04	0.13 ± 0.02	0.13 ± 0.04
Total glutathione	nmol/gFW	515 ± 57	428 ± 32	465 ± 66
Oxidised glutathione	GSSG/GSH ratio	0.034 ± 0.006	0.049 ± 0.012	**0.069 ± 0.008**
Detoxifying enzymes				
GST-CDNB	mU/g FW	102 ± 3	178 ± 41	**235 ± 32**
GST-Fluorodifen	mU/g FW	4.1 ± 0.5	5.9 ± 1.1	6.2 ± 1.1
GST-Npa	mU/g FW	21 ± 2	**34 ± 1**	35 ± 6
Membrane damage				
TBA reactive molecules	nmol/gFW	30 ± 3	33 ± 2	37 ± 4
Potassium leakage	% extracellular K	5.8 ± 0.3	7.7 ± 1.4	5.1 ± 1.4
Pigment and nutrient profile				
Pigment profile				
Chlorophyll *a*	mg/gFW	1.1 ± 0.2	1.4 ± 0.1	1.5 ± 0.1
Chlorophyll *b*	mg/gFW	0.63 ± 0.08	0.82 ± 0.06	0.81 ± 0.06
Chl *a/b*		1.9 ± 0.02	1.7 ± 0.1	1.8 ± 0.2
Total Chlorophyll	mg/gFW	1.8 ± 0.2	2.2 ± 0.1	2.3 ± 0.2
Carotenoids	mg/gFW	0.16 ± 0.01	0.16 ± 0.02	0.23 ± 0.04
Chl / Car		11 ± 1	15 ± 3	11 ± 2
Nutrient content				
Macronutrients				
K	(mg/gDW)	30 ± 1	31 ± 0.4	**39 ± 1**
Ca	(mg/gDW)	20 ± 1	21.2 ± 0.3	**26 ± 1**
P	(mg/gDW)	12 ± 0.3	13 ± 0.5	**15 ± 0.3**
S	(mg/gDW)	11 ± 0.3	12 ± 0.2	12 ± 1
Mg	(mg/gDW)	3.7 ± 0.2	3.9 ± 0.1	4.4 ± 0.2
Micronutrients				
Na	(mg/gDW)	1.2 ± 0.1	1.3 ± 0.1	1.0 ± 0.04
Fe	(µg/gDW)	438 ± 104	489 ± 275	171 ± 19
Mn	(µg/gDW)	76 ± 3	82 ± 1	**137 ± 7**
Zn	(µg/gDW)	29 ± 5	29 ± 3	41 ± 2
Cu	(µg/gDW)	4.2 ± 0.4	4.4 ± 0.1	**7.2 ± 0.3**

Response level	Unit	28 Days after metazachlor treatment Metazachlor treatment 0 mM	0.2 mM	0.4 mM
Growth				
Shoot weight	g	9.7 ± 0.8	**5.5 ± 1.1**	**2.6 ± 0.6**
Root weight	g	2.8 ± 0.6	**0.9 ± 0.3**	**0.2 ± 0.1**
Shoot length	cm	4.7 ± 0.6	**2.5 ± 0.2**	**1.6 ± 0.1**
Root length	cm	29 ± 2	**19 ± 1**	**12 ± 1**
Antioxidant defence and detoxification				
Total antioxidative capacity	µmol Trolox eq./gFW	24 ± 1	27 ± 2	28 ± 1
Hydrophilic fraction AO	µmol Trolox eq./gFW	18 ± 1	18 ± 2	18 ± 1
Lipophilic fraction AO	µmol Trolox eq./gFW	5.7 ± 0.7	**9.1 ± 0.9**	**10 ± 0.4**
Antioxidative enzymes				
SOD	mU/g FW	261 ± 18	305 ± 13	**441 ± 32**
CAT	mU/g FW	13 ± 3	27 ± 1	**37 ± 6**
APx	U/g FW	8.4 ± 2	12 ± 1	14 ± 3
GR	mU/g FW	394 ± 6	**516 ± 40**	**644 ± 63**
SPx	mU/g FW	108 ± 35	177 ± 24	169 ± 15
GPx	mU/g FW	35 ± 15	43 ± 6	**112 ± 25**
Antioxidative metabolites				
Total ascorbate	µmol/gFW	0.88 ± 0.20	0.95 ± 0.17	0.95 ± 0.15
Oxidised ascorbate	DHA/ASA ratio	0.15 ± 0.07	0.61 ± 0.18	0.50 ± 0.18
Total glutathione	nmol/gFW	276 ± 148	151 ± 15	148 ± 14
Oxidised glutathione	GSSG/GSH ratio	0.028 ± 0.014	0.027 ± 0.010	0.035 ± 0.013
Detoxifying enzymes				
GST-CDNB	mU/g FW	69 ± 19	116 ± 11	**148 ± 19**
GST-Fluorodifen	mU/g FW	3.0 ± 0.6	5.0 ± 0.8	**7.2 ± 1.4**
GST-Npa	mU/g FW	22 ± 1.0	28 ± 2	**34 ± 2**
Membrane damage				
TBA reactive molecules	nmol/gFW	23 ± 2	**32 ± 2**	**33 ± 3**
Potassium leakage	% extracellular K	8.4 ± 1.6	5.6 ± 1.4	14 ± 2
Pigment and nutrient profile				
Pigment profile				
Chlorophyll *a*	mg/gFW	0.81 ± 0.02	**0.95 ± 0.05**	0.89 ± 0.03
Chlorophyll *b*	mg/gFW	0.24 ± 0.003	0.27 ± 0.01	0.27 ± 0.01
Chl *a/b*		3.5 ± 0.1	3.6 ± 0.1	3.3 ± 0.1
Total Chlorophyll	mg/gFW	1.05 ± 0.06	**1.22 ± 0.06**	1.15 ± 0.03
Carotenoids	mg/gFW	0.145 ± 0.004	0.164 ± 0.008	**0.162 ± 0.004**
Chl / Car		7.2 ± 0.1	7.4 ± 0.2	7.1 ± 0.4
Nutrient content				
Macronutrients				
K	(mg/gDW)	56 ± 5	47 ± 2	45 ± 1
Ca	(mg/gDW)	26 ± 2	30 ± 2	30 ± 2
P	(mg/gDW)	7.3 ± 0.2	**4.6 ± 1.5**	**6.1 ± 0.3**
S	(mg/gDW)	14 ± 1	15 ± 1	14 ± 1
Mg	(mg/gDW)	5.5 ± 0.2	**7.0 ± 0.2**	**7.1 ± 0.3**
Micronutrients				
Na	(mg/gDW)	1.4 ± 0.1	**2.7 ± 0.1**	**2.3 ± 0.2**
Fe	(µg/gDW)	204 ± 25	295 ± 62	230 ± 58
Mn	(µg/gDW)	101 ± 18	155 ± 10	114 ± 30
Zn	(µg/gDW)	39 ± 5	**60 ± 3**	**16 ± 5**
Cu	(µg/gDW)	4.3 ± 0.5	4.3 ± 0.1	3.5 ± 0.2

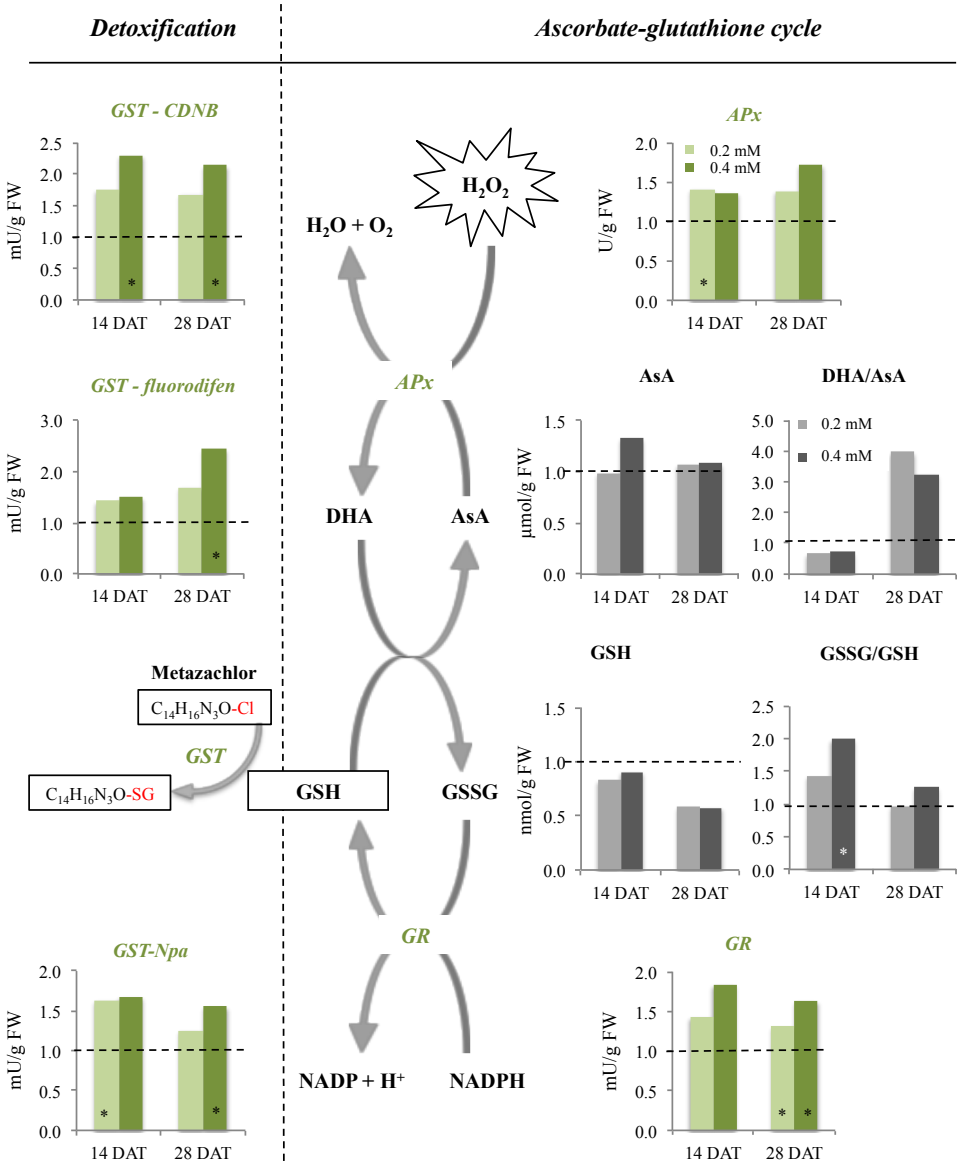

Figure 4.5. *An overview of the relative enzyme activities (green) and metabolite concentrations (grey) that play a role in either the detoxification of herbicides or the antioxidant defence mechanism. Data are expressed relative to control values (dashed line). Abbreviations used: APx (ascorbate peroxidase), AsA (ascorbate), CDNB (chlorodinitrobenzene), GST (glutathione S-transferase), DHA (dehydroascorbate), GSSG (glutathione disulphide), GSH (glutathione), GR (glutathione reductase), Npa (nitrophenylacetate), NADPH (Nicotinamide adenine dinucleotide phosphate). (* post-hoc value $p < 0.05$)*

Fourteen-day-old plants exposed to 0.4 mM metazachlor contained higher nutrient levels in their aboveground areal parts. Levels of macronutrients, such as potassium, calcium and phosphorus, as well as of micronutrients, such as manganese and copper, increased significantly within a range of 25 to 80% under the influence of 0.4 mM metazachlor (Table 4.1). Twenty-eight DAT, the nutrient profile in metazachlor-treated plants was different from control plants, with significant losses of phosphorus and significant augmentation of magnesium and sodium (Table 4.1).

4.5. Discussion

In the present study, controlled growth experiments of oilseed rape were carried out using microcosms in temperature-, light- and moisture-controlled growth chambers. This experimental set-up enabled us to investigate the specific impact of metazachlor on the crop *B. napus*, with exclusion of the complex interaction with soil characteristics and soil organisms, the direction and rate of drainage and the presence of herbivores, such as snails. Hence, the underlying mechanisms that ultimately determine the degree of phytotoxicity could be studied.

4.5.1. The detoxification metabolism in *B. napus* is activated two to four weeks after metazachlor application

The detoxification capacity of a crop is crucial for the neutralisation of xenobiotic compounds and ultimately determines the potential harm induced by that compound, either in form of direct interaction or via the induction of oxidative stress. The presence of metazachlor in the aboveground organs of young oilseed seedlings pointed out that metazachlor was taken up by roots and translocated into the shoots within two weeks after application. The decrease of the internal metazachlor concentration in the aboveground parts of *B. napus* during the subsequent weeks indicated the activation of the detoxification metabolism (Figure 4.1B). The rate of detoxification decreased with increasing metazachlor dose two weeks after application, which might indicate that the detoxification metabolism is either suppressed by the high internal metazachlor concentration or that it reached its maximal turnover (Figure 4.1B). Taking into account the rate of detoxification, the results of this laboratory test set up suggest that metazachlor might be entirely metabolised internally within 10 to 12 weeks after

application (Figure 4.1B). This result is in line with reported half-lives of metazachlor in the soil, that range between 19 to 82 days (Mamy *et al*. 2008). In general, the detoxification rate of herbicides is determined by the activity of cytochrome P450 peroxidases, GSH and GST. The detoxification of chloroacetamides does not involve phase I metabolisation by cyt P450 and is only facilitated by GST-mediated conjugation (Coleman *et al.* 1997). However, the tolerance of a crop towards a certain herbicide is not solely determined by the activity of GST. Glutathione concentration and redox state are also important (Hatton *et al.* 1996). After metazachlor application, the increased activity of GST at both time points and the increment in GSSG fraction at 14 DAT suggest that the detoxification of metazachlor was activated in oilseed rape (Figure 4.5). Two weeks after metazachlor application, the increased activity of GST with affinity towards CDNB and Npa substrates indicates that metazachlor and its metabolites were being conjugated with GSH prior to storage in the cell wall or in the vacuole (Figure 4.5). Four weeks after application, GST showed affinity to all tested GST substrates (Figure 4.5). Phi class GST's are closely associated with detoxification of chloroacetamides (Cho and Kong 2007). Since fluorodifen has been associated with tau-class GST activity (Jo *et al.* 2011) and CDNB is considered as a non-specific substrate (Cole 1994), it can be considered that both tau and phi classes of GSTs, which are most important routes for detoxification in plants (Thom *et al.* 2002), are involved in detoxification of metazachlor. Since the glutathione redox state is promoted towards its oxidised form (GSSG) under metazachlor treatment, GSH biosynthesis might not be able to provide the demand for GSH at this time point (Table 4.1). Two weeks later, the augmented activity of GST indicates that glutathione is still actively consumed for metazachlor-conjugation at this time point (Figure 4.5). In accordance with our results, the related chloroacetamide metolachlor induced a 5-fold increase in GST activity in maize (Viger *et al.* 1991). Although Viger *et al.* (1991) did not observe any changes in GSH content, Štajner *et al.* (2001) noticed decreased GSH contents in lettuce, pea and bean seeds under the influence of chloroacetamides, alachlor and metolachlor.

4.5.2. Metazachlor-induced oxidative stress results in membrane damage of plant cells

The presence of ROS, such as H_2O_2, in leaves of metazachlor exposed seedlings could not be revealed with the 3,3'-diaminobenzidine staining technique. However, the increased activity of enzymes involved in antioxidative defence and cell wall lignification and the activation of the AsA-GSH cycle, indirectly suggest the induction of pro-oxidants under the influence of metazachlor, two and four weeks after application (Table 4.1 and Figure 4.5). The total antioxidative capacity in leaf cells, determined by the ferric reducing antioxidative capacity (FRAP), comprises both water-soluble antioxidants, such as GSH, AsA, proline, phenolic compounds, membrane-bound molecules, and water-insoluble antioxidants, such as carotenoids and tocopherols (vitamin E) (Prior *et al.* 2005). The increase of the lipophilic fraction of antioxidants and the increase of carotenoid concentration at 28 DAT (Table 4.1), suggest a potential role for tocopherols as antioxidative compounds at this time point. Tocopherols have a significant role in herbicide-induced oxidative stress because of their ability to protect membrane-localised polyunsaturated fatty acids against ROS-induced lipid peroxidation (Fryer 1992, Munné-Bosch 2005). However, the simultaneous increase in lipid peroxidation suggests insufficient protection of leaf tissue against oxidative stress. Membrane integrity was estimated via potassium leakage and lipid peroxidation. The latter was significantly induced by metazachlor at 28 DAT (Table 4.1). Whereas the destabilisation of membranes can directly be induced through the inhibition of fatty acid biosynthesis by metazachlor, this also can be the result of metazachlor-induced oxidative stress. That these responses in membrane destabilisation became significant after four weeks could either be explained by the fact that the inhibition of VLCFA is a relatively slow process (Dayan and Watson 2011) or the fact that oxidative damage is a secondary side-effect of metazachlor. In general, herbicide-induced oxidative stress has been described in several crops (Gill and Tuteja 2010, Faheed 2011) and in particular also for chloroacetamides, such as alachlor and metolachlor (Štajner *et al.* 2001, Liu *et al.* 2012). The induction of the antioxidative enzymes SOD, APX, CAT and GR at two and four weeks after metazachlor application indicated metazachlor-induced oxidative stress (Table 4.1). The reduced CAT activity in the highest metazachlor treatment at

14 DAT could be linked to the high phytotoxic effects of metazachlor. However, the high sensitivity of CAT towards high levels of H_2O_2 has been described in different crop species under influence of various stresses, such as copper (Luna *et al.* 1994), herbicides (Song *et al.* 2007) and high and low temperatures (Feierabend *et al.* 1992). The high activities of cell wall bound peroxidases that use syringaldazine and guaiacol as substrates (Table 4.1) and that are involved in lignin biosynthesis, suggest either the apoplastic presence of ROS or the activation of cell wall lignification (Bestwick *et al.* 1998, Lee *et al.* 2007). Increased cell wall lignification could result in a reduced permeability by the settlement of a physical barrier and can therefore allow the cell to better protect itself against xenobiotic compounds. Lignin is known to be responsive to a range of stresses. Biotic and abiotic stresses (such as metals), are known to induce lignification in the walls of cells that do not lignify under non-stress responses (Wang *et al.* 2013), however this has not been described yet for herbicides. The increased activity of ascorbate peroxidase (APx) under the influence of 0.2 mM metazachlor at 14 DAT indicates that H_2O_2 is being converted actively into water and oxygen (Figure 4.5) and suggests the activation of the AsA-GSH cycle. This assumption is supported by the significant shift of the glutathione redox state towards its oxidised form, glutathione disulphide (GSSG), at 14 DAT and the increased activity of GR at 28 DAT under the influence of metazachlor (Figure 4.5). Together with glutathione's shift towards it's oxidised form, the increased activity of GST implies that GSH is consumed in the detoxification metabolism of metazachlor. Therefore, it can be presumed that GSH fulfils a dual role in both antioxidative defence and detoxification (Figure 4.4). Although ROS are known to induce oxidative damage, they also have an important function in signalling (Mittler *et al*. 2004). Hydrogen peroxide has been shown to regulate GST *in vivo* (Levine *et al*. 1995) and can therefore influence the rate of detoxification. GST induction by ROS would appear to represent an adaptive response as these enzymes detoxify some of the toxic carbonyl-, peroxide-, and epoxide-containing metabolites produced within the cell by oxidative stress (Hayes and Pulford 1995).

4.5.3. Metazachlor inhibits growth and induces fasciation the short term

Phytotoxic effects of metazachlor on oilseed rape became apparent shortly after its application, with a reduction of germination (Figure 4.1A), the manifestation of fasciation (Figure 4.2A) and the occurrence of mortality (data not shown). Fasciation of the leaves was already induced immediately after seedling emergence (Figure 4.2b). Typical symptoms of chloroacetamide herbicides, such as stunted growth, cupped and wrinkled leaves, shortened main veins and leaf fasciation were induced and were formerly observed in *Arabidopsis thaliana* exposed to related chloroacetamides, acetolachlor, alachlor and metolachlor (DeRidder and Coldsbrough 2006). Metazachlor-induced fasciation could be attributed to the mode of action of chloroacetamides, whereby the inhibition of VLCFA synthesis has led to the inhibition of normal cell division (Böger *et al.* 2000, Wu *et al.* 2000). During the further development of the crop, stem and shoot weight of the emerged seedlings was suppressed by metazachlor (Figure 4.3). Reductions in crop shoot length (Table 4.1) have previously been observed in *Sorghum sp*. under the influence of the chloroacetamide, metolachlor (Wilkinson and Duncan 1993). Although the timing of appearance of the leaves of *B. napus* seedlings seemed not to be influenced by metazachlor (Figure 4.4), the surface of the leaves and the petiole length were noticeably reduced (Supplementary file A). This could be linked with an insufficient capacity of light capitation for photosynthesis and therefore with the reduction of shoot weight. However, the pigment profile was not influenced as such (Table 4.1). In contrast to previous studies where pigment content was negatively affected by pesticides (Le Yin *et al.* 2008, Serra *et al.* 2014), chlorophyll and carotenoid concentrations rather tended to increase under the influence of metazachlor (Table 4.1). Although, metazachlor did not have any effect on the rate of leaf development up to five weeks after treatment, metazachlor-exposed plants tended to develop leaves faster than control plants between five and seven weeks after treatment (Figure 4.4). Taking into consideration the development of leaves and the similar weights of metazachlor exposed and non-exposed seedlings nine weeks after application (Figure 4.3C), the seedlings appeared to recover from the initial herbicide stress by investing in leaf development.

4.6. Conclusions

In conclusion, this controlled microcosm experiment demonstrated that, on the short term, metazachlor induces significant adverse effects on oilseed rape at morphological level. Together with an induction of the detoxification metabolism and the activation of the antioxidative defence responses, a reduction in growth investment was observed. These observations underpin the hypothesis that plants are investing energy in detoxification of the absorbed metazachlor and in the neutralisation of metazachlor-induced ROS, over shoot growth. This strategy seems to suffice for the plants to recover, as nine weeks after application their weight did not differ any more from the non-treated plants. When considering the weight of the aboveground areal plant parts, the metazachlor-exposed plants appear to be able to recover from the initial chemical-induced stress.

4.7. References

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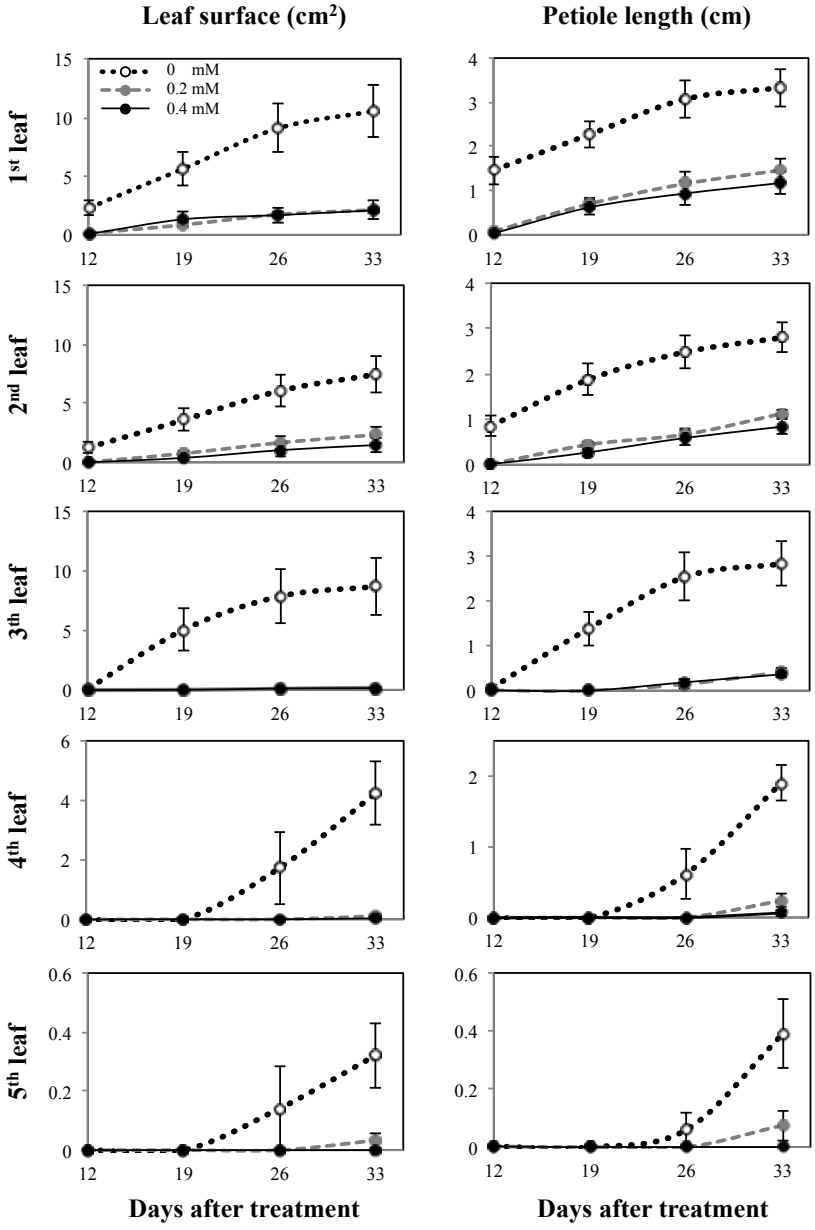
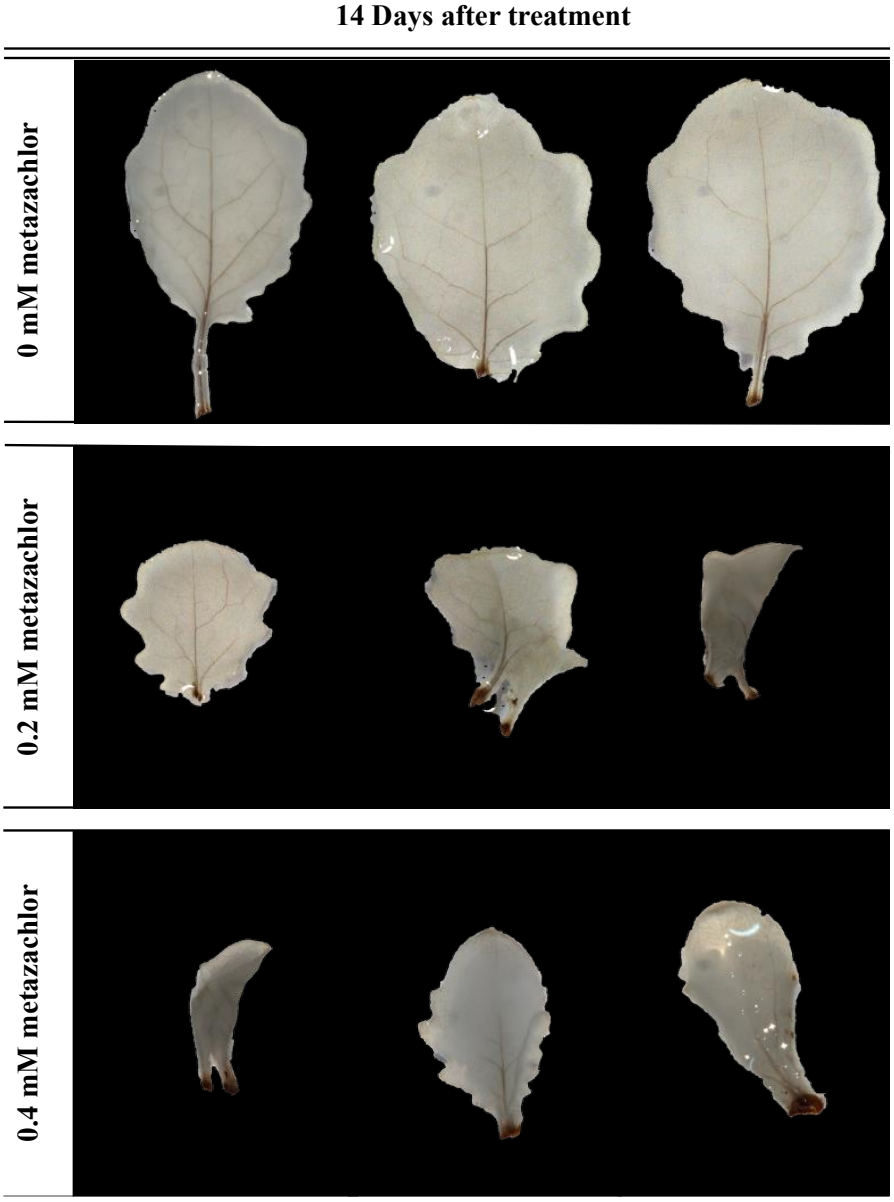
4.8. Supplementary files

Supplementary file A. *Leaf development of* B. napus *monitored weekly, after pre-emergent application with* 0 mM (dotted line), 0.2 mM (dashed line) and 0.4 mM (solid line) metazachlor*. Leaf surface (a) area and petiole length (b) were measured at 12, 19, 26 and 33 DAT.*



Supplementary file B. *Qualitative determination of H_2O_2 in leaves of* B. napus*, 14 days after metazachlor application (0 mM, 0.2 mM, 0.4 mM) by 3,3'-diaminobenzidine staining according to Daudi et al. (2012).*



Chapter 5 Elucidating the tolerance of *Brassica napus* to metazachlor by studying the balance between detoxification and antioxidative processes and the occurrence of phytotoxicity after post-emergent application

5.1. Abstract

This study reports about the acute effects of post-emergent soil-applied metazachlor on *B. napus.* Metazachlor was rapidly, *i.e.* within 24 h after treatment (HAT), taken up from the soil by the roots and was readily translocated to the aboveground parts. Initially, within 24 HAT, the increased activities of GST and antioxidative enzymes in the leaves indicate an activation of direct metazachlor detoxification on the one hand and detoxification of metazachlor-induced reactive oxygen species (ROS) on the other hand. Nevertheless, distortion of the cell membrane could not be avoided at this time point, leading to leakage of potassium. After 72 h of exposure, metazachlor uptake was augmented and induced increased amounts of H_2O_2 in leaves. This indicated the disturbance of the detoxification metabolism of metazachlor and hence insufficient removal of metazachlor-induced ROS. The decrease in GST activity, together with the stagnation of antioxidative enzyme activities suggested that time was needed for recovery of the enzymes involved in detoxification and antioxidative defence. In parallel, cell membranes became destabilised or damaged, which could be related either to the direct action of unmetabolised metazachlor or indirectly by metazachlor-induced ROS formation. In the aboveground plant parts the morphological effects also became apparent within the first week after treatment, leading to fasciation of the leaves and stagnation of growth. Whereas treated plants seemed to catch up in their development with control plants within seven weeks after treatment, their fresh weight did not restore within this time period.

5.2. Introduction

The success of herbicide application in agricultural practices is based on the selective phytotoxic actions of the xenobiotic compound towards desired and undesired plant species. Besides differences in effect on plant morphology, dose and mode of application and herbicide uptake, this selectivity strongly depends on the chemical characteristics of the xenobiotic compound on the one hand and to the intrinsic ability of the plant to withstand chemical stress on the other hand, also referred to as the tolerance of the plant (Marrs 1996, Pinto de Carvalho *et al.* 2009, Cobb and Reade 2010). Due to their chemical properties, different xenobiotic compounds have the ability to affect a range of targets

within the plant, such as photosynthesis, biosynthesis of amino acids and proteins or cell division. The specific sensitivity of such a target process within a certain plant species contributes to the selective capacity of the xenobiotic compound. The tolerance of exposed plants towards chemical stress is determined by their capacity to directly neutralise the xenobiotic compound and to maintain the redox balance by their antioxidative defence mechanisms. The different rate of herbicide degradation between crops and weeds is a major determinant for herbicide selectivity (Andrews *et al.* 1997). The rate of detoxification depends on the relative reactivity of the xenobiotic compound (Phase I), the bioavailability of the thiol substrate glutathione (GSH) and the presence of glutathione S-transferases (GST) (phase II) and finally the sequestration and catabolism of the resulting conjugate (Phase III) (Marrs 1996). To protect plant cells from injury, all steps of the detoxification process should be coordinated to run at rates that can effectively deal with the xenobiotic influx into the cell (Coleman *et al.* 1997). GST conjugation is assumed to be a crucial step in determining the rate of xenobiotic neutralisation and hence the tolerance of a species (Schröder and Collins 2002). As such, the induced activity of GST under the influence of the pharmaceutical acetaminophen in *Brassica juncea* L. leaves within a time span of 24 to 72 h after application was attributed towards its role in xenobiotic detoxification (Bartha *et al.* 2010). Besides, GSTs are also known to play a role in scavenging reactive oxygen species (ROS) as an antioxidant enzyme against a range of abiotic stresses (Armstrong 1991, Hayes and Pulford 1995). For example, drought stress induced the increment of GST activities in different *Brassica* species within 48 h after initiating the stress (Alam *et al.* 2014). Xenobiotics, such as herbicides, are known to induce the production of ROS (Le Yin *et al.* 2008, Radetski *et al.* 2000). Reactive oxygen species are assumed to fulfil a dual role during abiotic stress responses; either by cell injury through oxidative power and as key regulators in defence pathways (Møller and Sweetlove 2010). The balance between both outcomes is subtle and is under tight control of the metabolic and enzymatic antioxidants (Miller *et al.* 2008). The capacity of a plant to maintain its internal redox state stable is crucial for cell homeostasis and hence for normal cell functioning. Previously, comparative studies have focussed on the role of GST and antioxidants in the determination of differences

in herbicide tolerance between crops and weeds (Lao *et al.* 2003, Islam *et al.* 2015). Under the influence of atrazine and fluorodifen, the activities of antioxidant enzymes and GST were higher in wheat than in ryegrass, which was reflected in sub-lethal effects in the latter in the form of increased lipid peroxidation levels and reduced growth (Del Buono *et al.* 2011). Based on the occurrence and concentrations of different metabolites of an acetolactate synthase (ALS) inhibiting herbicide, oilseed rape has been assumed to posses a different mechanism of detoxification than the sensitive weed, crickweed (Yue *et al.* 2012). Hatton *et al.* (1996) revealed that the higher activity of GST in maize over its associated weeds species resulted in a higher tolerance against chloroacetamide herbicides. In agriculture, the development and application of safeners on agricultural crops is based on the augmentation of GST activity (Liu *et al.* 2009). As such, Cottingham and Hatzios (1991) have measured a 35% increase of GST activity and GSH content in maize after treatment with a safener.

In the agronomic practice of *B. napus* cultivation, metazachlor is applied either pre- or post-emergent (BASF, Fytoweb). Post-emergent application must be performed before the growth stage of nine leaves (GS19) is reached. In addition, the study of the cellular status of leaf cells closely after this time point of application can provide more insights in the immediate responses to herbicidal stress. Since the tolerance of a species towards herbicides mainly relies on its detoxification capacity and on efficiency of the antioxidant defence mechanisms, we hypothesise that these processes are important in the determination of the extent of phytotoxicity and tolerance closely after exposure. Therefore, this study focuses on the immediate responses after metazachlor application on *B. napus* and on the further development of the plants up to seven weeks after treatment. Hereby, the temporal significance of plant processes involved in detoxification and preservation of the cellular redox state during seedling development were studied by monitoring key enzymes and metabolites involved therein.

5.3. Experimental design and methodology

Three days before sowing, *B. napus* (cultivar *Remy*) seeds were surface sterilised. Seeds were washed in a 0.1% sodium hypochlorite solution for 2 min and subsequently rinsed thoroughly with deionised water. Next, seeds were rinsed in deionised water during 20 min and afterwards stored in a closed Petri dish on a moistened filter. After being incubated in the dark at 4 °C during two nights, seeds were separately sown in microcosms on 0.1625 kg sandy soil at 1 cm depth. In each microcosm three seeds were sown. Fourteen days after the seeds were sown and the plants were situated in growth stage 11-12, 10 mL of metazachlor solution was applied on the soil surface in the following concentrations: 0 mM, 0.2 mM and 0.4 mM metazachlor, which corresponded with 0 mg, 0.5 mg and 1 mg active ingredient per plant. Every two days, 10 to 50 mL ½ Hoagland nutrient solution was applied on the sandy surface. Plants were grown in a climate chamber under controlled environmental conditions: 12 h photoperiod, 65% relative humidity and day/night temperatures of 22 °C and 18 °C, respectively. A combination of blue, red and far-red LED modules simulated the photosynthetic active radiation (PAR) of 200 µmol $m^{-2}s^{-1}$ of sunlight at plant level. Seven days after sowing, the number of plants was reduced to one plant per microcosm. Leaf tissue for biochemical analyses was collected 24 and 72 HAT and subsequently stored at -70 °C. During sampling, biomass, root and shoot length were measured. All biochemical analyses were conducted as described in Chapter 2. A long-term experiment of nine weeks was conducted to follow up growth. Thereby, the growth stage of each individual plant was determined according to Lancashire *et al.* (1991). The set-up of this experiment was equal to the experimental set-up described in Chapter 4 (§ 4.3).

5.4. Results

5.4.1. Metazachlor uptake, growth and development of B. napus

Metazachlor was detected in the aboveground biomass of *B. napus* 24 and 72 HAT. The uptake of metazachlor increased with time and was dose-dependent, with 0.57 and 1.14 mg metazachlor kg^{-1} FW present in the aboveground biomass of 0.2 and 0.4 mM metazachlor-treated plants at 24 HAT, respectively (Figure 5.1A). Two days later, the internal metazachlor concentration increased with 39% and 25% in both treatments, resulting in 0.79 and 1.43 mg metazachlor kg^{-1} FW in *B. napus* under influence of the respective treatments. A growth curve was derived from daily stem length measurements, displaying the relative increase of stem length (Figure 5.1B). This growth curve shows that soon after treatment, within 24 HAT, the increase of stem length was inhibited by the highest metazachlor dose (0.4 mM). This effect became more pronounced 72 HAT and can be related with the increased metazachlor uptake in the aboveground plant parts. Concurrently, the development of the crop was suppressed after metazachlor application (Figure 5.2). Although the

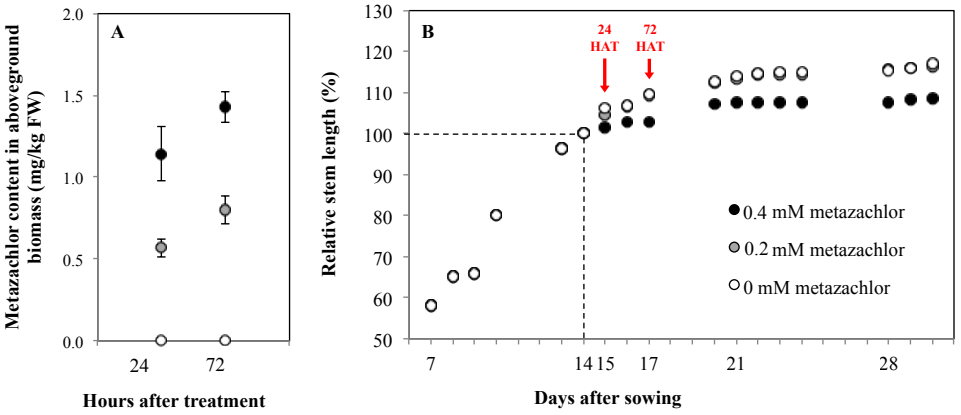


Figure 5.1. (A) *Concentration of metazachlor (mg kg^{-1} fresh weight) present in the aboveground biomass of* B. napus*, 24 and 72 HAT (indicated with arrows) with 0 mM (°), 0.2 mM (•□ and 0.4 mM (•□ metazachlor. Data are represented as average values of minimal 3 biological replicates ± SE.* (B) *Growth curve of* B. napus *treated with 0 mM (°), 0.2 mM (•□□and 0.4 mM (•□ metazachlor at day 14, based on daily stem length measurements. Data are represented as relative values with reference to day 14 (dotted line), at which plants had an average stem length of 3.57 cm (= 100%). Data are represented as average values of 10 biological replicates ± SE.*

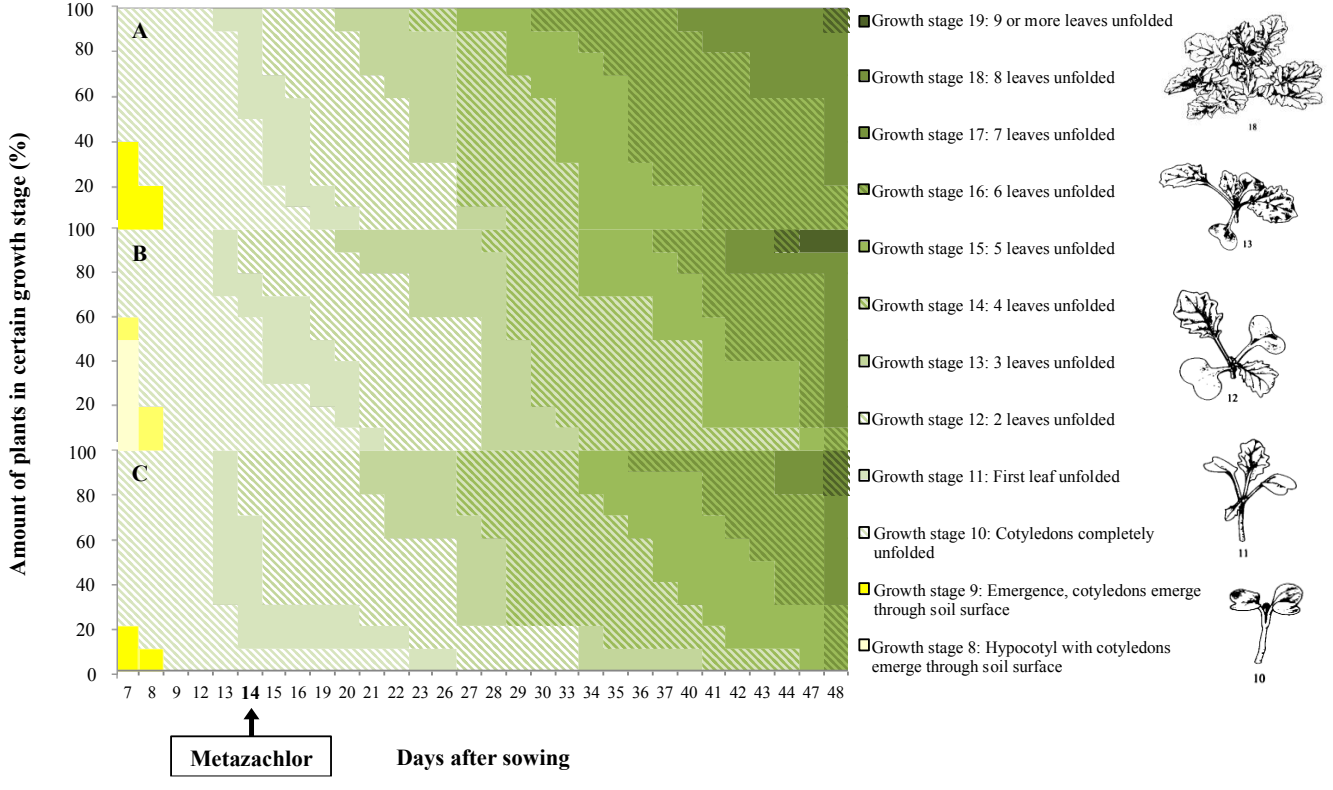


Figure 5.2. *Emergence (yellow) and leaf development (green) of* B. napus *over time, treated with (A) 0 mM, (B) 0.2 mM and (C) 0.4 mM metazachlor at day 14, expressed as the amount (%) of all measured plants that are situated in a specific developmental stage at a certain time point. Each condition contained 10 biological replicates. Different growth stages are depicted as described by Lancashire et al. 1991.*

development of plant leaves in different treatments was comparable during the first two weeks, plants post-treated with metazachlor stayed longer in an early growth stage after exposure. Consequently, progression in development was hampered as compared to control plants. The delay in development of the leaves became apparent two weeks after application (28 days after sowing). For example, three weeks after application (35 days after sowing) all control plants had developed 5 to 6 leaves (growth stages 15 and 16), while the majority of the metazachlor-exposed plants developed 3 to 4 leaves (growth stage 14 or lower) (Figure 5.2). Almost five weeks after treatment (48 days after sowing), no differences in development were observed in plants from the different conditions (Figure 5.2), indicating that within two months after metazachlor-treatment plants had catched up with leaf development of control plants. Considering the root and shoot biomass in terms of growth, it can be stated that growth was inhibited already shortly after treatment (Figure 5.3A, Table 5.1). Whereas shoot biomass was comparable for the different treatments at 24 HAT, root and shoot biomass were lower under influence of metazachlor at 72 HAT. Seventy-two HAT shoot biomass of 0.2 to 0.4 mM metazachlor-exposed plants was 10 to 28 % lower in comparison to control plants, respectively (Figure 5.3B and Table 5.1).

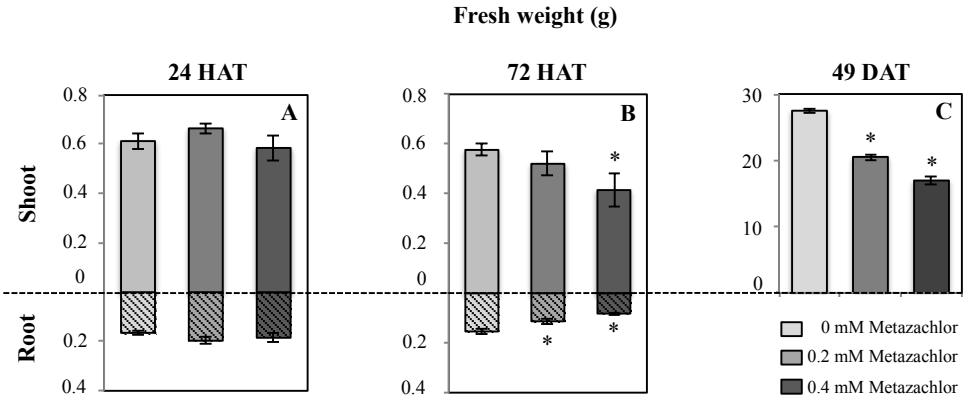


Figure 5.3. *Shoot and root fresh weight of* B. napus *(A) 24 HAT, (B) 72 HAT and (C) 49 DAT exposed to 0 mM (light grey), 0.2 mM (grey) and 0.4 mM (dark grey) metazachlor. Data are represented as average values of 10 biological replicates ± SE. (* post-hoc values: $p < 0.05$)*

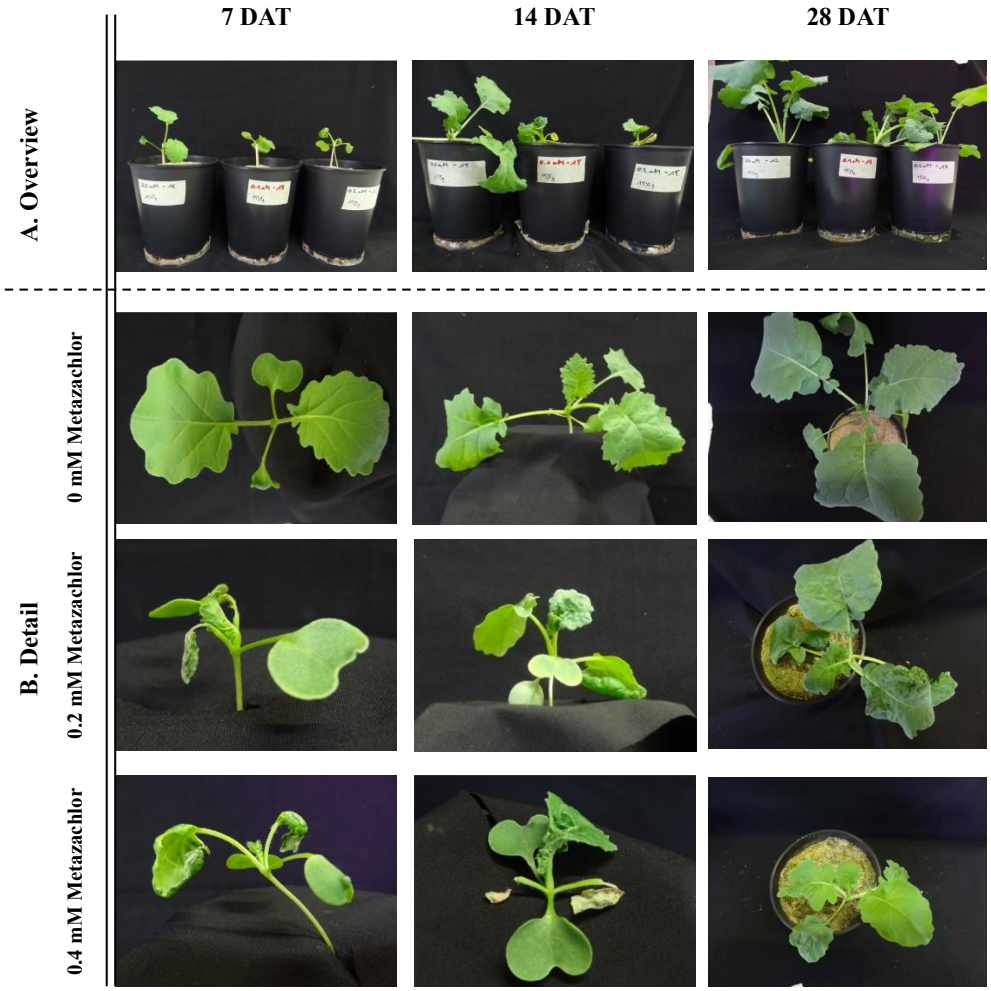


Figure 5.4. *(A) Overview images of* B. napus *exposed to 0, 0.2 and 0.4 mM metazachlor at 7, 14 and 28 DAT (i.e. 21, 28 and 42 days after sowing, respectively). (B) Detailed images of* B. napus *exposed to 0, 0.2 and 0.4 mM metazachlor at 7, 14 and 28 DAT. Plants exposed to 0.2 mM and 0.4 mM metazachlor exhibited malformation of the leaves.*

Root weight of herbicide-treated plants was even more suppressed with 26 to 47% at the respective doses (Figure 5.3B and Table 5.2). In the longer term, seven weeks after application (49 DAT), these differences between the different treatments were still present (Figure 5.3C). Figure 5.4 presents both overview and more detailed pictures of *B. napus* under different treatments over time. The first signs of metazachlor-induced morphological malformations became

apparent 7 DAT (Figure 5.4B). Fourteen DAT, 80 and 100% of the plants treated with 0.2 and 0.4 mM metazachlor displayed signs of fasciation, respectively (data not shown). Malformations ranged from uncompleted separation of leaves, curling of leaf edges, curved leaf surfaces and leaf nerves.

5.4.2. Enzymes and metabolites involved in detoxification

The total level of GSH, a metabolite that acts as a substrate during GST-mediated herbicide conjugation remained unaffected under the influence of metazachlor at 24 and 72 HAT. Twenty-four HAT the activity of GST was higher in metazachlor-exposed plants towards the substrates chlorodinitrobenzene (CDNB), fluorodifen and nitrophenylacetate (Npa) compared with control plants (Table 5.1). No activity of GST towards the substrates 1,2-dichloro-4-nitrobenzene (DCNB), 4-nitrobenzyl chloride (NBC) and p-nitrobenzpyl chloride (NBoC) was observed. The activity of CDNB-GST was significantly higher as compared to controls with a 1.9 and 2.3-fold higher activity after application of 0.2 mM and 0.4 mM metazachlor, respectively. Seventy-two HAT, the activity of GST was lower in metazachlor-treated plants in comparison with control plants towards all substrates, with a significant reduction in GST-CDNB activity in 0.2 mM metazachlor-treated plants at 72 HAT (Table 5.2).

5.4.3. Metazachlor induces oxidative stress in the leaves of *B. napus*

Qualitative determination of hydrogen peroxide with 3,3'-diaminobenzidine revealed an overall presence of H_2O_2 in leaf cells of the first developed leaf 72 HAT (Figure 5.5). The intensity of staining was irrespective of the applied metazachlor concentration and was only present in the oldest leaves. The total antioxidant capacity in leaf cells was not influenced by metazachlor neither at 24 HAT nor at 72 HAT. At the latter time point however, the antioxidative capacity of the lipophilic fraction was 1.7 times higher under the influence of 0.4 mM metazachlor. In addition, the activities of enzymes involved in antioxidative defence (SOD, CAT, APx and GR) and cell wall lignification (GPx and SPx) were

Table 5.1. *Parameters concerning metazachlor uptake, growth, antioxidant defence, detoxification metabolism, membrane damage and pigment profile in* B. napus *leaves, 24 HAT with 0 mM, 0.2 mM and 0.4 mM metazachlor. Data are shown as average values of minimal 4 biological replicates ± SE. * Significant decreases and increases are accentuated in light grey and dark grey, respectively, with post-hoc values: p < 0.05.*

Response level	Unit	24 Hours after metazachlor treatment		
		Metazachlor treatment (mM)		
		0	0.2	0.4
Metazachlor uptake				
Metazachlor concentration	mg/kg FW	0.00 ± 0.00	0.57 ± 0.05	1.14 ± 0.17
Growth				
Shoot weight	mg	614 ± 32	665 ± 23	586 ± 51
Root weight	mg	165 ± 11	197 ± 13	185 ± 17
Stem length	cm	4.0 ± 0.2	3.4 ± 0.3	3.7 ± 0.2
Root length	cm	11.5 ± 0.4	11.3 ± 0.5	11.7 ± 0.5
Antioxidant defence and metabolisation				
Total antioxidative capacity	µmol Trolox eq./gFW	22 ± 1	22 ± 5	18 ± 4
Hydrophilic fraction AO	µmol Trolox eq./gFW	19 ± 1.3	18 ± 4	15 ± 4
Lipophilic fraction AO	µmol Trolox eq./gFW	2.9 ± 0.4	3.5 ± 1.0	3.2 ± 0.3
Antioxidative enzymes				
SOD	mU/g FW	254 ± 32	343 ± 67	**500 ± 71**
CAT	mU/g FW	28 ± 9	31 ± 6	45 ± 10
APx	U/g FW	15 ± 2	16 ± 1	20 ± 1
GR	mU/g FW	484 ± 51	665 ± 61	700 ± 44
SPx	mU/g FW	67 ± 25	66 ± 18	104 ± 0.3
GPx	mU/g FW	212 ± 47	211 ± 24	275 ± 40
Antioxidative metabolites				
Total ascorbate	µmol/gFW	5.5 ± 0.6	4.5 ± 0.5	5.3 ± 0.5
Oxidised ascorbate	DHA/ASA ratio	0.21 ± 0.01	0.32 ± 0.10	0.32 ± 0.07
Total glutathione	nmol/gFW	355 ± 32	339 ± 77	318 ± 16
Oxidised glutathione	GSSG/GSH ratio	0.051 ± 0.007	0.067 ± 0.015	0.061 ± 0.019
Detoxifying enzymes				
GST-CDNB	mU/g FW	123 ± 15	**232 ± 21**	**288 ± 32**
GST-Fluorodifen	mU/g FW	3.4 ± 1.27	4.7 ± 1.43	3.1 ± 0.4
GST-Npa	mU/g FW	16 ± 3	25 ± 3	22 ± 8
Membrane Damage				
TBA reactive molecules	nmol/gFW	18 ± 2	18 ± 1	16 ± 2
K leakage	% extracellular K	5.4 ± 0.5	**10.1 ± 0.8**	10.0 ± 2.0
Pigment profile				
Chlorophyll a	mg/gFW	0.79 ± 0.01	0.79 ± 0.01	0.75 ± 0.02
Chlorophyll b	mg/gFW	0.15 ± 0.002	**0.13 ± 0.004**	**0.13 ± 0.01**
Chl a/ Chl b		5.2 ± 0.04	**6.0 ± 0.2**	**5.9 ± 0.1**
Total Chlorophyll	mg/gFW	0.94 ± 0.02	0.92 ± 0.01	0.87 ± 0.03
Carotenoids	mg/gFW	0.46 ± 0.03	**0.56 ± 0.03**	0.52 ± 0.03
Chl/ Car		2.1 ± 0.1	**1.7 ± 0.1**	**1.7 ± 0.1**

Table 5.2. *Parameters concerning metazachlor uptake, growth, antioxidant defence, detoxification metabolism, membrane damage and pigment profile, in* B. napus *leaves 72 HAT with 0 mM, 0.2 mM and 0.4 mM metazachlor. Data are shown as average values of minimal 4 biological replicates ± SE. * Significant decreases and increases are accentuated in light grey and dark grey, respectively, with post-hoc values: p < 0.05.*

Response level	Unit	72 Hours after metazachlor treatment		
		Metazachlor treatment (mM)		
		0	0.2	0.4
Metazachlor uptake				
Metazachlor concentration	mg kg FW^{-1}	0.00 ± 0.00	0.79 ± 0.09	1.43 ± 0.09
Growth				
Shoot weight	mg	1153 ± 49	1041 ± 93	**830 ± 132**
Root weight	mg	308 ± 20	**227 ± 21**	**163 ± 11**
Stem length	cm	4.1 ± 0.2	3.6 ± 0.2	3.8 ± 0.2
Root length	cm	13.4 ± 0.2	13.6 ± 0.7	11.7 ± 0.4
Antioxidant defence and metabolisation				
Total antioxidative capacity	µmol Trolox eq./gFW	15 ± 2	13 ± 2	18 ± 1
Hydrophilic fraction AO	µmol Trolox eq./gFW	13 ± 2	11 ± 2	14 ± 1
Lipophilic fraction AO	µmol Trolox eq./gFW	2.5 ± 0.5	2.5 ± 0.3	4.3 ± 0.8
Antioxidative enzymes				
SOD	mU/g FW	438 ± 43	327 ± 20	**202 ± 18**
CAT	mU/g FW	49 ± 6	42 ± 10	63 ± 5
APx	U/g FW	12 ± 1	15 ± 2	15 ± 2
GR	mU/g FW	583 ± 22	542 ± 46	526 ± 21
SPx	mU/g FW	101 ± 13	126 ± 10	63 ± 11
GPx	mU/g FW	243 ± 31	245 ± 35	145 ± 25
Antioxidative metabolites				
Total ascorbate	µmol/gFW	4.3 ± 0.7	4.0 ± 0.5	4.4 ± 0.4
Oxidised ascorbate	DHA/ASA ratio	0.29 ± 0.10	0.24 ± 0.05	0.34 ± 0.06
Total glutathione	nmol/gFW	237 ± 24	317 ± 31	306 ± 28
Oxidised glutathione	GSSG/GSH ratio	0.092 ± 0.030	0.048 ± 0.010	0.071 ± 0.018
Detoxifying enzymes				
GST-CDNB	mU/g FW	230 ± 26	**157 ± 18**	185 ± 14
GST-Fluorodifen	mU/g FW	2.4 ± 0.4	1.2 ± 0.1	1.2 ± 0.1
GST-Npa	mU/g FW	25 ± 2	22 ± 2	22 ± 1
Membrane Damage				
TBA reactive molecules	nmol/gFW	13 ± 2	17 ± 2	14 ± 1
K leakage	% extracellular K	7.3 ± 0.7	**16.2 ± 0.8**	**28.3 ± 3.4**
Pigment profile				
Chlorophyll a	mg/gFW	0.82 ± 0.02	**0.72 ± 0.02**	0.77 ± 0.04
Chlorophyll b	mg/gFW	0.14 ± 0.01	0.12 ± 0.01	0.14 ± 0.01
Chl a/ Chl b		6.1 ± 0.3	6.1 ± 0.3	5.7 ± 0.2
Total Chlorophyll	mg/gFW	0.95 ± 0.03	**0.84 ± 0.02**	0.91 ± 0.06
Carotenoids	mg/gFW	0.64 ± 0.02	0.63 ± 0.05	**0.55 ± 0.01**
Chl/ Car		1.5 ± 0.1	1.4 ± 0.1	1.7 ± 0.1

determined. The activities of all antioxidant enzymes tended to be higher after metazachlor application in a dose-dependent manner in comparison with non-treated plants at 24 HAT (Table 5.1). This trend was statistically supported for superoxide dismutase (SOD) activity, that was nearly 2 times higher in 0.4 mM metazachlor-exposed plants than in control plants (Table 5.1). However, the activities of the antioxidant enzymes tended to be similar or lower in 0.2 and 0.4 mM metazachlor-treated plants after 72 h of exposure, which was only supported statistically for SOD (Table 5.2). Neither the levels of the antioxidant metabolite ascorbate (AsA) nor its redox state were altered by metazachlor at both sampling points.

5.4.4. *Metazachlor induces membrane leakage and shifts in pigment profile*

Thiobarbituric acid-reactive metabolites were present in leaf tissue in equal amounts at 24 and 72 HAT in all conditions, suggesting that there was no increase of lipid peroxidation in leaf cells due to metazachlor application (Tables 5.1, 5.2). However, the significant increases of extracellular potassium concentrations under the influence of metazachlor at both time points suggest the impairment of leaf cell membranes (Tables 5.1, 5.2). Twenty-four HAT, the extracellular concentrations of potassium were two-fold higher in plants treated with 0.2 and 0.4 mM metazachlor, regardless of the concentration applied. Seventy-two HAT, the increases were two- and four-fold under influence of 0.2 and 0.4 mM metazachlor, respectively. The pigment profile differed between treatments within 24 HAT. At this time point, the chlorophyll *b* concentration was 13% lower in both 0.2 and 0.4 mM metazachlor-treated plants in comparison with control plants, which resulted in a 15% lower chlorophyll *a/b* ratio, independent of the applied dose. Carotenoids were more abundant in 0.2 and 0.4 mM metazachlor-treated plants, with a significant higher content of carotenoids in 0.2 mM metazachlor-treated plants. Seventy-two hours after application, chlorophyll *a* content was 12% lower in 0.2 mM-treated plants and carotenoid concentration was 14% lower under the influence of 0.4 mM metazachlor in comparison with non-treated plants.

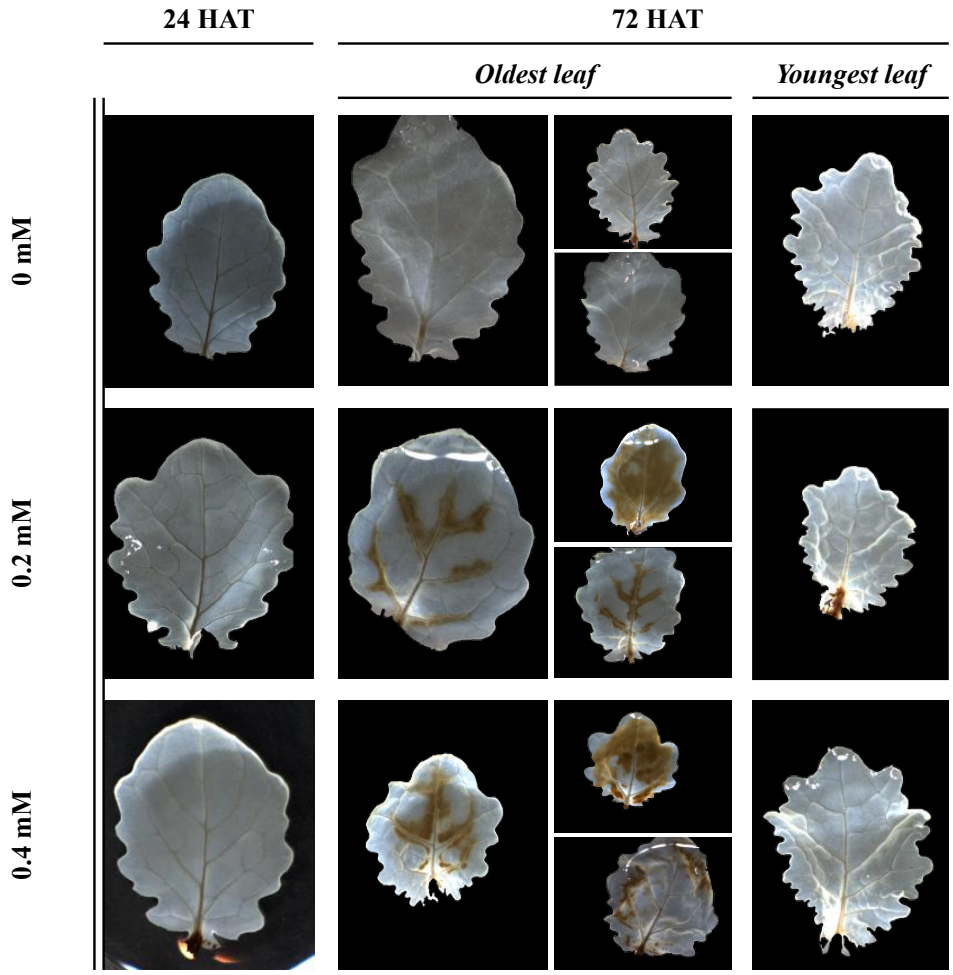


Figure 5.5. *Qualitative determination of H_2O_2 in leaves of* B. napus*, 24 and 72 hours after metazachlor treatment (HAT) (0, 0.2, 0.4 mM) by 3,3'-diaminobenzidine staining according to Daudi et al. (2012). At 24 HAT no brown coloured complex between 3,3'-diaminobenzidine and H_2O_2 was present, suggesting the absence of H_2O_2. At 72 HAT, the brown colour points out the presence of H_2O_2 in the oldest leaves, represented by 3 biological replicates. No H_2O_2 was present in the newly formed leaves at 72 HAT.*

5.5. Discussion

In order to investigate the immediate plant responses to herbicide application, we applied metazachlor in a post-emergent manner on the soil surrounding the young seedlings. This not only allowed us to investigate the immediate cellular responses within days after application, the timing of application was also in line with field practices. A one-factorial microcosm test was set up under controlled environmental conditions to investigate the sole impact of the herbicidal application to the plant. Since literature indicates that shoots are more susceptible towards abiotic disturbance (Cottingham and Hatzios 1991) and because these tissues ultimately serve as by-product in animal feed production, the focus of this study was set on the aboveground plant tissue.

5.5.1. Detoxification of metazachlor is activated closely after exposure

The rate at which the detoxification metabolism is activated within a plant species is assumed to be a major determinant in herbicide selectivity (Cobb and Reade 2010). Moreover, it will initially determine the intensity of herbicide-induced damage that is either directly induced through the action of the herbicide or indirectly through the herbicide-induced generation of ROS. Herbicides from different chemical classes have been shown to induce ROS formation within plants (Obermeier 2015). As an agricultural crop, *B. napus* is assumed to cope with herbicides at rates that are sufficient to prevent lethal crop damage. However, the cellular mechanisms behind this capacity are not uncovered yet. As the metazachlor solution was applied on the sandy substrate surrounding the young crop plant, the occurrence of metazachlor in aboveground plant parts 24 HAT indicates that metazachlor was rapidly taken up by the root and translocated to the aerial plant parts. In addition, the increases of internal metazachlor concentrations in the aboveground plant parts within the following days suggested that the rate of uptake and root to shoot translocation is higher than the rate of herbicide detoxification. The accumulation of internal metazachlor within this small timeframe coincided with a shift in activity of key components of the detoxification and antioxidative defence metabolism.

The increased activity of GST towards the universal substrate CDNB in metazachlor-treated plants at 24 HAT suggests a fast and specific activation of phase II detoxification (Table 5.1). In accordance with our results, the related chloroacetamide metolachlor induced a 5-fold increase in GST activity in maize after 10 min of exposure (Viger *et al.* 1991). In contrast to earlier studies, clear differences in GST activity patterns between substrates were observed (Tables 5.1, 5.2). This suggests a time-dependent action of differential GST isoforms. Nutricati *et al.* (2006) have shown that phi class gene expression is enhanced in *Arabidopsis thaliana* after 24 h exposure to different chloroacetamide herbicides, alachlor and metolachlor. Since corresponding phi class proteins showed activity towards CDNB (Nutricati *et al.* 2006), our results indicate an increased activity of phi class GSTs within 24 HAT. Within the next two days, GST activity significantly declined in the presence of metazachlor (Table 5.2). The reduction of GST activity towards CDNB substrates in treated plants at this time point can be the result of either direct impairment by metazachlor, indirect impairment via metazachlor-induced ROS or by reaching the maximal turnover capacity of GST. Based on the absence of a correlation between an increment of GS-conjugates and growth inhibition of oat exposed to related chloroacetamides, alachlor, metolachlor and propachlor, Böger *et al.* (2000) assumed that these compounds could not be considered as inhibitors of SH-containing enzymes. However, Schröder and Collins (2002) found evidence that GS-conjugates, which probably are formed shortly after application in our experimental setup, might inhibit GST activities. GST is known to be induced by H_2O_2 in the response to oxidative stress (Levine 1994). Based on this, the dual role of H_2O_2, *i.e.* signalling versus damaging, comes forward in the present study. Whereas an initial limited cellular rise of H_2O_2 can lead to activation of CDNB-based GST, the increased levels of H_2O_2 at 72 HAT (Figure 5.5) concomitantly with a time- and dose-dependent rise in membrane damage (K-leakage: Tables 5.1, 5.2) could explain the damaging effect of metazachlor-induced oxidative stress and hence the fall in activity of GST. Because of the fast response of GST activity towards herbicide exposure, further investigations into the regulation of different classes of GST are required and could contribute to the development of new herbicides and selection of varieties.

5.5.2. Metazachlor induces an oxidative burst in leaves within 72 h of exposure

The increased activities of antioxidative enzymes, such as SOD, in the leaves of *B. napus* indicated the occurrence of excess ROS 24 h after the application of metazachlor. However, at this time point, the antioxidant defence seemed to be sufficient to prevent an oxidative burst as the 3,3'-diaminobenzidine staining of the first appeared leaf did not indicate excessive presence of H_2O_2 (Figure 5.5). This observation is in line with the early activation of GST-mediated detoxification and hence prevention of metazachlor-induced ROS production. This corresponds to the signalling function of H_2O_2 suggested for GST activities and the tight control of its concentrations by antioxidative enzymes. Two days later (72 HAT), the opposite, decreasing trend in activity of antioxidative enzymes coincided with the damaging responses and clearly elevated H_2O_2 concentrations observed in leaves (Figure 5.5). It is noteworthy that the presence of H_2O_2 was only observed in the first developed, *i.e.* oldest, leaves which suggests a difference in susceptibility between developed and developing leaves. The locally induced presence of H_2O_2 might be explained by the probability that young, developing leaves contain higher levels of GST and possess a higher protein turnover and thus a higher detoxification capacity. Findings of Gullner *et al.* (2001) support this hypothesis by revealing that GST activity was highest in developing leaves of poplar trees, indicating that the age of an organ is important in determining its capacity to efficiently detoxify xenobiotic compounds. However, in this study no distinction was made between activities of key molecules involved in detoxification and antioxidative defence among oldest and youngest leaves. Future studies concerning the timing of detoxification metabolism should incorporate the different susceptibility among younger and older plant organs.

To relate the induction of ROS with the efficiency of the crop to neutralise the electrophilic metazachlor, a selection of well-known antioxidative enzymes were measured in leaf tissue, together with the total antioxidative capacity, which includes both water-soluble (*e.g.* GSH, AsA, proline, phenolic compounds and membrane-bound molecules) and water-insoluble antioxidants (ß-carotene and tocopherols). No difference in total antioxidative capacity (TAC) of the leaves was observed at 24 HAT (Table 5.1). The lipophilic fraction of antioxidants was

higher 72 HAT of 0.4 mM metazachlor (Table 5.2). Furthermore, oxidative membrane damage, estimated by determining TBA reactive metabolites (Tables 5.1, 5.2), was not observed in our experimental setup and might be related to the higher lipophilic antioxidant capacity. However, the lower levels of carotenoid content in 0.4 mM metazachlor-treated plants 72 HAT suggest that the increase of lipophilic antioxidative capacity is probably mainly due to the action of tocopherols, since they are known to function as membrane-located antioxidants by protecting phospholipids from lipid peroxidation (Havaux *et al.* 2005). Nevertheless, the leakage of potassium after metazachlor application indicates that cell membranes are destabilised most likely due to the working mechanism of metazachlor. As a chloroacetamide, metazachlor interferes with enzymes involved in very long chain fatty acid biosynthesis, which are major compounds in cellular membranes (Fuerst 1987).

5.5.3. Metazachlor induces a retardation in growth of *Brassica napus*

The fast uptake and translocation of metazachlor to the aboveground plant parts led to immediate stagnation of root and stem growth and hence plant weight (Figures 5.1 and 5.3). Within seven days after metazachlor application severe distortions of leaf morphology in the form of fasciated leaves became apparent (Figure 5.4). This can be explained by the working mechanism of metazachlor, whereby disruption of VLCFA biosynthesis can result in membrane impairment and hence cell division distortion (Deal and Hess 1980, Weisshaar and Böger). Especially during the development of young plants, the balance between developmental pathways and detoxification and defence is very delicate and thus susceptible for xenobiotic disturbance (Suzuki *et al.* 2012, Kocsy *et al.* 2013). Young *et al.* (2003) observed that the age of the plant at exposure also determines the degree of phytotoxicity responses, with plants younger than 30 days being more susceptible towards herbicide exposure.

5.6. Conclusions

In conclusion, this study reveals that after post-emergent application of metazachlor the active ingredient is rapidly taken up by the roots and quickly translocated into the aerial parts of the plant. Despite the early activation of metabolites and enzymes involved in detoxification and antioxidative defence, membrane instability could not be prevented and a stagnation of plant growth occurred. Together with the increase of metazachlor in leaves in time, H_2O_2 was observed in the oldest leaves and an imbalance in the cellular redox state was induced. The retardation in growth and development under influence of metazachlor indicates that plants are not able to completely recover within two months after treatment. These observations support that the hours following application are very crucial in determining whether a plant can withstand the herbicidal treatment. The extent of herbicide-mediated ROS induction and the role of ROS in signalling versus damage should be further studied in leaves from different age to obtain a better understanding concerning the best time of application.

5.7. References

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Chapter 6 The functional role of the photosynthetic apparatus in the recovery of *Brassica napus* plants from pre-emergent metazachlor exposure

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6.1. Abstract

Metazachlor is a chloroacetamide herbicide, frequently used in *Brassica napus* cultivations around the world. Its primary target is the inhibition of very long chain fatty acid biosynthesis. This study included a morphological and physiological screening of hydroponically grown *B. napus*, exposed to a concentration range of 0, 0.25, 0.50, 0.75 and 1.0 kg metazachlor per hectare. The results indicate that within a month after application, growth and development of *B. napus* are severely affected by low metazachlor doses. At intermediate metazachlor concentrations, loss of phosphorus and potassium from the plant tissues suggests destabilisation of cellular membranes, which may be a direct consequence of metazachlor application. This membrane instability could be indirectly linked with alterations of electron transport and a reduction of carbon assimilation. At increased metazachlor doses of 0.75 kg a.i. ha^{-1}, pigment concentrations are strongly reduced. However, chlorophyll fluorescence parameters seem to remain unaffected at metazachlor doses up to 0.75 kg a.i. ha^{-1}. At a metazachlor concentration of 1.0 kg a.i. ha^{-1}, negative effects are observed on all tested parameters, resulting in limited survival. The results indicate photosynthesis is assured at intermediate metazachlor concentrations for the cost of growth and development. It is clear that photosynthesis plays a key role in the survival strategy of young plants to overcome initially induced chemical stress.

6.2. Introduction

The oilseed crop *Brassica napus* is increasingly gaining importance worldwide. Its global production has been doubled over the last ten years with main producers in Europe (36.5%) and Asia (37%) and (FAOSTAT 2013). The applications of its products are manifold. Seed-extracted oil is applied in food and biofuel industry and the residual press cake is used in fodder production. The most commonly used herbicide on oilseed crops is metazachlor belonging to the chloroacetamide herbicides (European Commission 2012), which inhibit the formation of very long chain fatty acids that play a key role in cell division and cell expansion processes (Fuerst 1987, Böger 2003). Herbicide selectivity is determined by the rate of plant cellular metabolism, where the active ingredient is detoxified into a less active glutathione conjugate (Cobb and Reade 2010). In

general, herbicides are applied as chemical control agents limiting the growth of weeds. Although the active herbicide ingredient is supposed to inhibit a weed-specific target site, (Cobb and Reade 2010), it may also induce stress to the crop plant itself (Mitra and Raghu 1998, Zaidi 2005, Le Yin *et al*. 2008). Phytotoxicity induced by chloroacetamide herbicides has been described on different biological organisation levels. At morphological level, crops were reported to display stunted growth (Foy and Witt 1990) and leaf deformation (Dhillon and Anderson 1972). In addition, growth reduction of broadleaved tree seedlings under metazachlor treatment has been observed by Willoughby *et al.* (2006). At plant physiological level, propachlor, another member of the chloroacetamide herbicides, has been observed to reduce contents of chlorophylls and carotenoids in barley (Kleugden 1980). Chlorophyll fluorescence is often used as a tool to evaluate crop production (Baker and Rosenqvist 2004) and as a measure for monitoring phytotoxicity in crops (Dvorak and Remesova 2001, Riethmuller-Haage *et al*. 2006, Jin *et al*. 2011, Buonasera *et al*. 2011). Photosynthesis can be affected by many abiotic stresses, such as salt (Moradi and Ismail 2007, Jafarinia and Shariati 2012), drought (Müller *et al*. 2010, Husen *et al*. 2014), light (Guidi and Degl'Innocenti 2012), ozone (De Bock *et al*. 2012), metals (Vassilev and Manolov 1999, Saenen *et al*. 2014, Vanhoudt *et al*. 2014) and herbicides (Barbagallo *et al*. 2003). Chlorophyll fluorescence analysis is a most often applied technique for the measurement of photosynthesis and can be related to CO_2 assimilation. A relationship that is established through electron transport that delivers ATP and NADPH required for photosynthetic carbon assimilation. Abiotic stress can alter this relationship by increasing the activity of alternative electron sinks, such as the Mehler reaction, photorespiration and cyclic electron transport (Murchie and Lawson 2013). The overall cell metabolism, which accounts for the detoxification of herbicides and repair processes, is driven by photosynthesis and plays an important role in determining the extent of stress experienced by the crop. Through the presence of effective repair mechanisms of thylakoid protein complexes, crops are able to deal with minor chemical stress and photo-inhibition (Aro *et al*. 2004). Abbaspoor *et al*. (2006) have suggested that chlorophyll fluorescence measurements can be used as a method to estimate the capacity of sugar beet to recover from the triazine herbicide, metamitron exposure. Magné *et al*.

(2006) described a link between the re-greening of vine leafs (*Vitis vinifera*) after herbicide treatment with the recovery of CO_2 fixation rate, plastid ultra-structure and pigment concentration. Therefore, measuring photosynthetic processes can provide indications on the recovery of plants from herbicide treatment.

Metazachlor's mode of application is based on the pre-emergent treatment of the surface soil, thereby preventing annual grasses and broadleaved weeds to settle. This study involves a dose-response study under controlled conditions to unravel the occurrence and the extent of the herbicide-induced phytotoxicity of the pre-emergent herbicide metazachlor on the non-target crop plant, *B. napus*, with particular attention to photosynthesis.

6.3. Experimental design

6.3.1. Plant material

In order to exclude soil-related influencing factors, plants were grown on a semi-hydroponic test-setup in which perlite was used as a substrate to provide stability to the plants. The plants were grown in a growth chamber, under controlled conditions: 250 µmol m^{-2} s^{-1} light intensity, 14/10 h photoperiod, 24/18 °C day/night temperature and constant relative humidity of 65%. *B. napus* (var. Remy) seeds were sown at 1 cm depth on a 5 cm thick perlite substrate, which was saturated with ½ Hoagland solution and replenished with fresh solution on a weekly basis. The day after sowing, metazachlor (Butisan S, BASF) was sprayed on the surface of the perlite substrate. The selection of the concentration range was based on the quantity and concentration of herbicide often used by farmers. According to the guidelines, it is permitted to apply 1.0 kg active ingredient, dissolved in 1000 L of water. In practice, however, often 1.25 kg active ingredient is dissolved in 400 L water, which corresponds with a concentration of 11.12 mM. Since the latter concentration was lethal for the plants in this hydroponic test system, the following concentration range was selected: 0, 2.25, 4.50, 6.75 and 9.00 mM which corresponded with 0, 0.25, 0.50, 0.75 and 1.0 kg active ingredient per hectare (kg a.i. ha^{-1}), with the highest dose in accordance with the recommended dose (BASF). One week after sowing, plant density was adapted manually to 30 plants per tray.

Photosynthetic analyses were performed in vivo 28 days after application of metazachlor. Thereafter, total fresh weight of the plant (root and shoot) was recorded and at the same time samples of fresh leaf material were taken and snap frozen in liquid nitrogen for the determination of metazachlor, lipid peroxidation, pigment profile and NPK profile.

6.3.2. Chlorophyll fluorescence and gas exchange analysis

Chlorophyll fluorescence and gas exchange measurements were performed on intact, dark- and light-adapted leaves with a pulse modulation fluorometer (MINI-PAM, Heinz Walz, Germany), before taking samples for biochemical assessment. Plants were kept in the dark for at least 25 min before measurement. By switching on the measuring beam (0.02 – 0.20 µmol m^{-2} s^{-1}), the minimal level of fluorescence (F_0) was recorded. Immediately thereafter, a saturating light pulse (SLP) of 5500 µmol m^{-2} s^{-1} with 0.8 s duration was sent out to record the maximal level of fluorescence in the dark-adapted state (F_m), from which the maximal quantum yield of PSII [F_v/F_m] was calculated (with $F_v = F_m - F_0$). After 30 min of light adaptation at 250 µmol m^{-2} s^{-1}, the steady-state level of photosynthesis was reached and a saturating light pulse (5500 µmol m^{-2} s^{-1}) was applied. Based on the measurements of fluorescence yield before the SLP (F) and the maximal fluorescence reached during the SLP (F_m'), following parameters were calculated: the effective yield of photochemical energy conversion [$Y = (F_m' - F) / F_m'$] and the electron transport rate [ETR = Y*PAR*0.5*0.84] (White and Critchley 1999). According to Schreiber (2004), photochemical quenching [$q_P = (F_m' - F) / (F_m' - F_0)$] and non-photochemical quenching [$q_N = (F_m - F_m') / (F_m - F_0)$] quenching parameters, as well as NPQ [$(F_m - F_m') / F_m$] were calculated.

In addition to the fluorescence parameters at steady-state photosynthesis, an estimation of electron transport rate (ETR) in light-adapted leaves was extracted from rapid light curve measurements. Rapid light curves were generated by sending out subsequent SPLs at different time intervals (10, 20, 30, 40, 50, 60, 70, 80 and 90 s after initiation). In order to determine additional photosynthetic parameters (ETR_{max}, α and E_k), ETR values were plotted against PAR values, and the Levenberg-Marquardt nonlinear least-squares model was used to fit the

correlation curve (Moré 1978). The maximal ETR (ETR_{max}) represents the maximal capacity of the electron transport chain to transfer electrons, α represents the photosynthetic rate in limited light conditions and E_k represents the minimal saturating irradiance, which can be calculated as ETR_{max}/α (Ralph and Gademann 2005).

The steady-state photosynthesis was determined by means of a closed gas-analytical system (Li-Cor, ADC Bioscientific ltd.). After plants and equipment had been adapted to surrounding light intensities during 20 min, stomatal conductance (gs), transpiration rate (E), CO_2 uptake (A) and intercellular to air CO_2 concentration (ci/A) were determined on intact leaves, under a light intensity of 250 µmol m^{-2} s^{-1} (PAR).

6.3.3. Plant sampling and biochemical assessment

Biometric analyses were performed 28 days after metazachlor treatment. Total fresh weight of the plant (root and shoot) was recorded. All biochemical analyses were performed on fresh leaf material. The biochemical analyses that were performed comprised the determination of the pigment profile, lipid peroxidation and nutrient analyses. Because this study involved evaluating the role of the photosynthetic apparatus in chloroacetamide-induced phytotoxicity, the focus was set on the aboveground shoot tissue, rather than root tissue. The techniques used for determination of lipid peroxidation and pigment profile were executed as described in Chapter 2 (§ 2.3 and § 2.5). Macronutrients, nitrogen (N), phosphorus (P) and potassium (K) were determined in plant leaves. Air-dried plant samples (500 mg) were mineralised with H_2SO_4 and H_2O_2 and finally dissolved in distilled water for further analyses. Total nitrogen content was determined according to the Kjeldahl method as described by Tomov *et al.* (1999). Phosphate content was determined according to Bergmann (1992). A blue-coloured complex, formed by the interaction between ammonium molybdate (NH_4MoO_4) and inorganic phosphate in the presence of $SnCl_2$, was measured at 720 nm. Potassium content was determined using flame photometry. Based on a standard curve of KCl, the percentage of KCl was determined. Biometric analyses could not be performed on plants treated with

the highest metazachlor dose, since these plants were too small to collect sufficient biomass.

6.3.4. Data analysis

Data were analysed by means of one-way ANOVA in open-source software R (R 3.1.2, The R Foundation for Statistical Computing, Vienna, Austria). Subsequently, Tukey post-hoc analysis was carried out to elucidate the pairwise comparison. In case data did not meet the conditions for normal distribution (Shapiro-Wilk cut-off ≤ 0.90), a non-parametrical Kruskal-Wallis test, followed by a post-hoc Wilcoxon test was performed. Parameters regarding ETR measurements were calculated from a fitted curve, based on the Levenberg-Marquardt nonlinear least-squares algorithm, generated in R (Moré 1978).

6.4. Results

6.4.1. Growth and metazachlor uptake

One month after application of increasing concentrations of metazachlor, the internal concentration of metazachlor in aboveground tissues increased from less than 0.5 to 3.4 mg kg^{-1} fresh weight (Figure 6.1A). The increase in metazachlor uptake in plant leaves was accompanied by a decrease of *B. napus* total weight

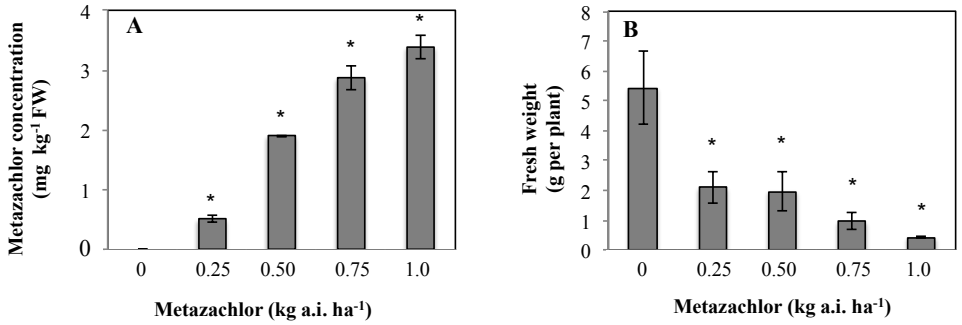

Figure 6.1. *(A) Metazachlor content in aboveground biomass and (B) total fresh weight of* B. napus *plants exposed to 0, 0.25, 0.50, 0.75 and 1.0 kg a.i. ha^{-1}. Biological replicates for metazachlor determination (N=2) were constituted from pooled samples (5 plants per control sample and 10-15 plants per treatment sample) and biological replicates for fresh weight determination were taken from individual samples (N=15, for all conditions). *Asterisks indicate significant difference with control values by post-hoc test at P < 0.05 level.*

(Figure 6.1B). The total fresh weight of the plants was strongly reduced, in a dose dependant way, and decreased between 62 to 92% compared to control plants (Figure 6.1B). Growth of *B. napus* was strongly suppressed under metazachlor treatment (Figure 6.2A) and leaves displayed signs of fasciation and chlorosis at the highest metazachlor treatment (1.0 kg a.i. ha^{-1}) (Figure 6.2B-C).

6.4.2. Biochemical responses

Concerning nutrient content of the aboveground tissues, N, P and K concentrations decreased along with increasing internal metazachlor content, indicating a loss of nutrients (Table 6.1). However, determination of TBA reactive metabolites did not indicate clear signs of lipid peroxidation under metazachlor treatment. Table 6.1 shows that chlorophyll *a*, chlorophyll *b* and overall carotenoid concentrations remained unaffected at low metazachlor doses up to 0.50 kg a.i. ha^{-1}. However, plants exposed to higher levels of metazachlor

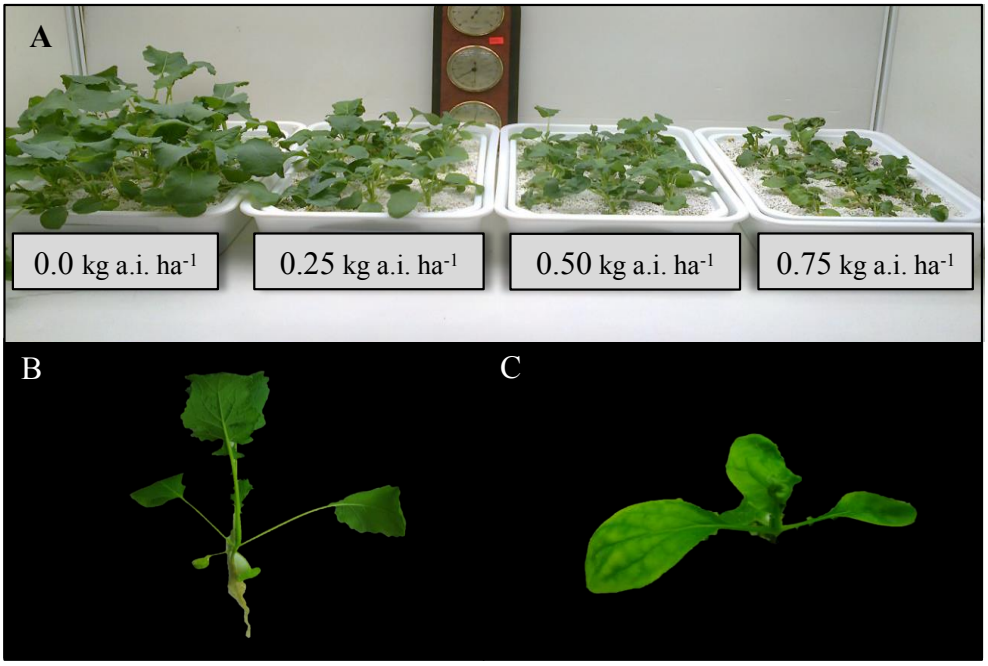

Figure 6.2. *An overview of the test experiment is pictured (A) and a detailed picture of 0 kg a.i. ha^{-1} (B) and 1.0 kg a.i. ha^{-1} (C) exposed plants are represented. Fasciation was clearly present in 1.0 kg a.i. ha^{-1} treated plants (C).*

Table 6.1. *Biochemical analyses in leaves of* B. napus*, 28 days after exposure to 0, 0.25, 0.50 and 0.75 and 1.0 kg metazachlor ha^{-1}. Data are presented as average values of minimal three biological replicates ± SE. * Significant decreases and increases are accentuated in light grey and dark grey, respectively, with post-hoc values: p < 0.05.*

		Metazachlor (kg a.i. ha^{-1})							
		0		0.25		0.50		0.75	
		value	%	value	%	value	%	value	%
Nutrient profile									
K	**(mg g^{-1} DW)**	5.46 ± 0.00	*100*	3.75 ± 0.01	*69* *	3.97 ± 0.01	*73* *	3.42 ± 0.02	*63* *
Nitrogen	**(mg g^{-1} DW)**	3.37 ± 0.03	*100*	3.57 ± 0.02	*106* *	3.60 ± 0.02	*107* *	3.08 ± 0.00	*92* *
P_2O_5	**(mg g^{-1} DW)**	1.23 ± 0.04	*100*	1.01 ± 0.01	*82* *	1.07 ± 0.01	*87* *	0.95 ± 0.01	*77* *
TBA reactive metabolites	**(nmol g^{-1}FW)**	23 ± 0.44	*100*	20 ± 0.99	*89*	25 ± 8.4	*107*	17 ± 2.30	*72*
Pigment profile									
Chlorophyll *a*	**(mg g^{-1}FW)**	1.36 ± 0.06	*100*	1.24 ± 0.04	*92*	1.42 ± 0.02	*105*	0.63 ± 0.11	*46* *
Chlorophyll *b*	**(mg g^{-1}FW)**	0.42 ± 0.07	*100*	0.46 ± 0.06	*108*	0.56 ± 0.01	*133*	0.35 ± 0.07	*82*
Total Chlorophyll	**(mg g^{-1}FW)**	1.77 ± 0.13	*100*	1.69 ± 0.01	*96*	1.98 ± 0.01	*112*	0.97 ± 0.18	*55* *
Carotenoids	**(mg g^{-1}FW)**	0.48 ± 0.01	*100*	0.44 ± 0.02	*92*	0.49 ± 0.01	*104*	0.22 ± 0.04	*47* *
Chl *a*/Chl *b*	**(mg g^{-1}FW)**	3.30 ± 0.40	*100*	2.78 ± 0.43	*84*	2.56 ± 0.10	*77*	1.83 ± 0.06	*55*
Tot Chl / Car	**(mg g^{-1}FW)**	2.24 ± 0.19	*100*	2.29 ± 0.13	*102*	2.55 ± 0.02	*114*	2.17 ± 0.18	*97*

had a different chlorophyll profile than non-treated plants. Total chlorophyll and overall carotenoid concentrations were reduced with 45% and 53%, respectively, in plants exposed to 0.75 kg metazachlor ha^{-1}. Since chlorophyll *a* was reduced three times more than chlorophyll *b*, the chlorophyll *a/b* ratio, which reflects the PSII to LHCII ratio (Kitajima and Hogan 2003), was reduced with 45%. The proportion of chlorophyll pigments over carotenoid pigments remained unchanged under metazachlor exposure.

6.4.3. Chlorophyll fluorescence

Chlorophyll fluorescence is a well known indicator for the functioning of the photosynthetic apparatus. The maximal quantum efficiency (Fv/Fm) of photosystem II (P680) was determined on dark-adapted leaves (Table 6.2). When treated with metazachlor concentrations up to 0.75 kg a.i. ha^{-1}, the maximal quantum efficiency was close to that of the control plants (0.82–0.83), indicating that the photosynthetic state of PSII remained unaffected. However, Fv/Fm was significantly reduced to 0.70 in crop plants exposed to the highest metazachlor dose of 1.0 kg a.i. ha^{-1}. This result suggests that the quantum yield of the primary photochemical reaction in the reaction center of PSII decreased by approximately 15%. This decrease is due to both an increase of F_0 and a decrease of Fm and consequent Fv. An increase of F_0 indicated low efficiency transfer of the trapped energy to the reaction centers, while the lower Fm may be linked to significant non-photochemical quenching in antenna of PSII (Table 6.2). In light-adapted leaves, results demonstrate that under the influence of the highest concentration, photochemical quenching (qP) decreased with 37% and non-photochemical quenching (qN) increased significantly with 79%. The significant increase of qN could be due to a retardation of oxygen evolving complex and forming of inactive quenching fluorescence state of the reaction center $P680^+$ of PSII (Strasser 1997). Maximal fluorescence in light-adapted leaves (Fm') and effective quantum yield (Y) remained steady in *B. napus* treated with metazachlor doses up to 0.75 kg a.i. ha^{-1}. However, Fm' was reduced with 30% when exposed to 1.0 kg metazachlor ha^{-1}, which resulted in a 57% decrease of effective quantum yield of PSII (Y).

Table 6.2. *Chlorophyll fluorescence parameters, measured on* B. napus*, 28 days after exposure to 0, 0.25, 0.50, 0.75 and 1.0 kg ha^{-1} metazachlor. Data are presented as average values of minimal three biological replicates ± SE. * Significant decreases and increases are accentuated in light grey and dark grey, respectively, with post-hoc values: p < 0.05.*

	Metazachlor (kg a.i. ha^{-1})									
	0		**0.25**		**0.50**		**0.75**		**1.0**	
	value	*%*	**value**	*%*	**value**	*%*	**value**	*%*	**value**	*%*
Dark-adapted leaves										
F0	224 ± 15	*100*	224 ± 3	*100*	205 ± 4	*91*	233 ± 17	*104*	259 ± 49	*116*
Fm	1236 ± 66	*100*	1241 ± 16	*100*	1193 ± 1	*97*	1313 ± 65	*106*	888 ± 218	*72*
Fv/Fm	0.82 ± 0.00	*100*	0.82 ± 0.00	*100*	0.83 ± 0.00	*101*	0.82 ± 0.00	*101*	**0.70 ± 0.05**	***85*** *
Light-adapted leaves										
F	508 ± 35	*100*	510 ± 8	*100*	485 ± 20	*96*	**581 ± 42**	***114*** *	465 ± 85	*92*
Fm'	797 ± 39	*100*	787 ± 22	*99*	735 ± 6	*92*	851 ± 15	*107*	**555 ± 103**	***70*** *
Y	0.36 ± 0.03	*100*	0.35 ± 0.01	*96*	0.34 ± 0.02	*94*	0.32 ± 0.05	*87*	**0.16 ± 0.05**	***43*** *
ETR	43 ± 2	*100*	39 ± 0.2	*89*	36 ± 3	*83*	**34 ± 3**	***80*** *	**18 ± 5**	***43*** *
qP	0.97 ± 0.004	*100*	0.92 ± 0.03	*95*	0.82 ± 0.14	*85*	0.81 ± 0.07	*84*	0.60 ± 0.18	*63*
qN	0.35 ± 0.05	*100*	0.29 ± 0.03	*84*	0.39 ± 0.01	*111*	0.30 ± 0.00	*85*	**0.63 ± 0.10**	***179*** *
NPQ	0.41 ± 0.08	*100*	0.32 ± 0.05	*79*	0.47 ± 0.02	*115*	0.32 ± 0.00	*80*	**1.48 ± 0.68**	***364*** *
ETRmax (electrons m^{-2} s^{-1})	91 ± 3	*100*	83 ± 0.01	*92*	82 ± 8	*91*	**68 ± 1**	***75*** *	**30 ± 9**	***33*** *
α	0.21 ± 0.01	*100*	0.20 ± 0.00	*98*	0.19 ± 0.01	*90*	0.19 ± 0.01	*92*	**0.13 ± 0.02**	***62*** *
Ek (µmol m^{-2} s^{-1})	433 ± 6	*100*	405 ± 2	*94*	434 ± 17	*100*	358 ± 27	*83*	252 ± 84	*58*

Table 6.3. *Gas exchange parameters measured on* B. napus*, 28 days after exposure to 0, 0.25, 0.50, 0.75 and 1.0 kg ha^{-1} metazachlor. Transpiration rate (E), stomatal conductance (gs), net CO_2 assimilation (A), intercellular CO_2 concentration (ci) and intercellular to net CO_2 assimilation (ci/A) were determined on intact leaves of B. napus, under light intensity of 250 µmol m^{-2} s^{-1} (PAR). Data are presented as average values of minimal three biological replicates ± SE. * Significant decreases and increases are accentuated in light grey and dark grey, respectively, with post-hoc values: p < 0.05.*

		Metazachlor (kg a.i. ha^{-1})											
		0		0.25			0.50			0.75			1.0		
		values	%	values	%		values	%		values	%		values	%	
E	**(mmol H_2O m^{-1} s^{-1})**	2.28 ± 0.06	*100*	2.67 ± 0.22	*117*		**2.64 ± 0.03**	***116***	*	2.33 ± 0.00	*102*		**0.74 ± 0.01**	***33***	*
gs	**(mol m^{-2} s^{-1})**	0.288 ± 0.013	*100*	**0.248 ± 0.014**	***86***	*	**0.333 ± 0.004**	***115***	*	**0.230 ± 0.000**	***79***	*	**0.034 ± 0.001**	***12***	*
A	**(µmol CO_2 m^{-2}s^{-1})**	13.9 ± 0.3	*100*	**12.4 ± 0.4**	***89***	*	**12.3 ± 0.4**	***88***	*	**11.4 ± 0.3**	***82***	*	**2.7 ± 0.2**	***19***	*
ci		278 ± 11	*100*	280 ± 6	*100*		279 ± 1	*99*		277 ± 3	*99*		354 ± 2	*126*	
A/ci		0.047 ± 0.002	*100*	0.045 ± 0.002	*95*		0.044 ± 0.001	*93*		0.042 ± 0.001	*88*		**0.007 ± 0.001**	***15***	*

The electron transport rate (ETR) was calculated from rapid light curve measurements in light-adapted leaves. At light intensities around 250-280 µmol m^{-2} s^{-1}, the ETR value in 0.50, 0.75 and 1.0 kg a.i. ha^{-1} exposed plants was significantly reduced, decreasing from 43 electrons $m^{-2}s^{-1}$ in control plantsto 37, 34 and 18 electrons $m^{-2}s^{-1}$ in the respective treatments (Table 6.2). The maximal ETR value in control leaves was 91 electrons $m^{-2}s^{-1}$ and was significantly reduced with 25 and 67% in 0.75 and 1.0 kg a.i. ha^{-1} exposed plants, respectively. The photosynthetic rate in light-limiting conditions (α) remained unaffected under the influence of metazachlor doses up to 0.75 kg a.i. ha^{-1} but was strongly inhibited at 1.0 kg a.i. ha^{-1} (Table 6.2). The PAR at which photosynthesis equals respiration rate (Ek), is significantly lower in plants treated with 0.75 (358 µmol m^{-2} s^{-1}) and 1.0 (252 µmol m^{-2} s^{-1}) kg a.i. ha^{-1} in comparison to control plants (433 µmol m^{-2} s^{-1}).

Additionally, gas exchange parameters were determined as a measure of steady-state photosynthesis (Table 6.3). With exception of the highest treatment, the effect of metazachlor on gas exchange parameters was ambiguous. Under the influence of 1.0 kg a.i. ha^{-1} the decrease in net CO_2 assimilation (A) could be addressed to mesophyll limitation and is supported by the observation of a decrease in stomatal conductivity (gs), CO_2 assimilation (A) and transpiration (E) occurring in parallel with an increased trend of intercellular CO_2 concentration (ci).

6.5. Discussion

To investigate the effects of the pre-emergent application of the herbicide metazachlor on the functioning of the photosynthetic apparatus in young emerging *B. napus* seedlings, a dose-response experiment was conducted. This study focussed on the relationships between the functioning of the photosynthetic apparatus, pigment content, nutrient content, leaf cell membrane integrity, and plant growth, at one month after application of metazachlor.

The increased metazachlor concentration in the aerial tissues was accompanied by a strong decrease in plant weight (Figure 6.1B), indicating that the uptake of low doses of metazachlor shortly after emergence induced severe effects on the biomass production of *B. napus*. Former field studies reported chloroacetamide-

induced crop injury in the form of stunting (Foy and Witt 1990) and yield reduction in broadleaved tree seedlings under metazachlor treatment (Willoughby *et al.* 2006). At morphological level, we found that metazachlor provoked apparent leaf structure deformation in *B. napus* (Figure 6.2C). Similar observations have been described in crops treated with another chloroacetamide herbicide, propachlor (Dhillon and Anderson 1972), and other classes of herbicides (Bondada 2011). The primary target of chloroacetamide herbicides involves the inhibition of very long chains fatty acid (VLCFA) biosynthesis, which could ultimately lead to the distortion of cell division in young weeds and crops by distortion of cell membrane structure (Matthes and Böger 2002, Böger 2003). Whereas metazachlor was present in leaf cells 28 days after treatment (Figure 6.1A), no indications of peroxidation of cell membranes lipids were found (Table 6.1). In contrast to this observation, the strong decreases in N, P, K concentrations in leaves indicated either a reduced nutrient uptake or the possible presence of membrane leakage (Table 6.1). In line with this, Jones *et al*. (2015) reported reductions of P and K contents in foliar tissue under the influence of pre-emergent herbicides. Severe distortion of VLCFA biosynthesis by metazachlor can lead to destabilisation of the lipid bilayers of membranes in the cell, thereby indirectly affecting the function of the photosynthetic apparatus, which is embedded in the thylakoid membranes of the chloroplast (Dayan and Zaccaro 2012). By measuring chlorophyll fluorescence in dark- and light-adapted leaves, pigment profile, electron transport rate and gas exchange, it was clear that with increasing exposure to metazachlor, different steps in the photosynthetic proces were affected (Tables 6.1, 6.2 and 6.3). The strong reductions of Fm and Fv/Fm in dark-adapted leaves together with the reduction of Y, Fm' and qP in light-adapted leaves clearly indicates that 1.0 kg a.i. ha^{-1} metazachlor treatment strongly inhibits photosynthesis in *B. napus* plants (Table 6.2). Maximal quantum yield (Fv/Fm) of photosystem II in crop plants exposed to 1.0 kg a.i. ha^{-1} metazachlor was strongly reduced (Table 6.2), mainly due to a reduction in Fm. In general, a reduction of fluorescence with respect to Fm can be associated with either enhanced photochemical energy conversion or by increased heat-dissipation (Maxwell and Johnson 2000). In this case, the lower Fm was linked with a significantly lower photochemical quenching (qP) and could

be a mechanism to avoid over-reduction of the primary electron acceptor of PSII, Q_A (Vassilev 2002).

Under exposure to 0.75 kg a.i. ha^{-1} metazachlor, the pigment profile of *B. napus* leaves was clearly altered (Table 6.1). The reduced chlorophyll content in 0.75 kg a.i. ha^{-1} exposed plants, resulted in chlorosis of the leaves. Strong reductions in chlorophyll *a*, chlorophyll *b* and carotenoids have not been reported before in *B. napus* exposed to metazachlor. However, Kleugden (1980) described a decreased pigment content in developing barley after exposure to the chloroacetamide propachlor. Whereas the pigment profile of *B. napus* was clearly disturbed by metazachlor, the quantum yield of photosystem II seemed to be mainly unaffected under exposure to metazachlor doses up to 0.75 kg a.i. ha^{-1} in both dark- and light-adapted leaves (Table 6.2). In dark-adapted leaves, the maximum quantum yield (Fv/Fm) was similar to that of control plants, indicating that the maximal potential efficiencies of PSII with all reaction centres open, were equal. Chen *et al*. (2007) have shown that the maximal quantum efficiency remained stable in the cyanobacterium *Nostoc* in response to the chloroacetamide butachlor. The fluorescence of light-adapted leaves exposed to doses up to 0.75 kg a.i. ha^{-1} metazachlor contained a comparable effective quantum yield (Y) as control leaves (Table 6.2). Hussain *et al*. (2010) found similar results in lettuce exposed to 1 mM metolachlor, a related chloroacetamide herbicide. It is noteworthy that under the exposure of 0.75 kg a.i. ha^{-1}, there was a tendency to a diminished non-photochemical quenching. This might indicate that excessive energy is less well quenched by photo-protective regulatory mechanisms, such as the xanthophyll cycle (D'Haese *et al*. 2004). Since xanthophyll cycle pigments are crucial for energy dissipation of excess absorbed light, the decreased carotenoid concentrations could account for this observation.

Whereas chlorophyll fluorescence was not affected by metazachlor doses up to 0.75 kg a.i. ha^{-1}, indicating that the efficiency of PSII was not altered, downstream electron transport was adversely affected at low metazachlor doses, from 0.50 kg a.i. ha^{-1} on, in light-adapted leaves (Table 6.2). Rapid light curves indicated that ETR_{max} was inhibited with 25 to 67% at exposure to 0.75 and

1.0 kg a.i. ha^{-1} doses, implying that ETR was very sensitive to metazachlor. Concurrently, the electron transport rate was limited at lower radiation values (Ek) with increasing exposure. These results indicate that less energy was conducted to PSI, which ultimately resulted in a reduced number of electrons for carbon fixation and other assimilation processes (Wodala *et al*. 2012). Dayan and Zaccaro (2012) have reported that carbetamide, a herbicide that targets VLCFA synthase, induces a 50% reduction of ETR, which could probably be explained as an effect of membrane destabilisation. The reduced electron transport rate in leaves of *B. napus* exposed to metazachlor concentrations of 0.50 kg a.i. ha^{-1} and higher, could be linked with a reduction of the net assimilation rate of CO_2 and hence a reduction in biomass production under these treatments. Nevertheless, the suppression of CO_2 assimilation was not accompanied by a decrease in captured excitation energy at 0.50 and 0.75 kg a.i. ha^{-1} metazachlor doses. Therefore, the excessive electrons that ran through PSII and that were not used in electron transport nor in non-photochemical quenching, would have to be stored in alternative electron sinks such as the Mehler reaction, photorespiration and cyclic electron transport (Xia *et al*. 2006, Takahashi *et al*. 2008).

In conclusion, it can be stated that *B. napus* plants were suffering from severe reductions in growth and development one month after the pre-emergent application of recommended metazachlor concentrations, which was reflected by a strong reduction of seedling weight at the low metazachlor dose of 0.25 kg a.i. ha^{-1} (Figure 6.1). At cellular level, metazachlor-induced damage was noticeable at multiple parameters. At an intermediate metazachlor dose of 0.50 kg a.i. ha^{-1}, the lower K, N and P contents suggested the occurrence of membrane leakage, which could be caused by metazachlor-inhibited biosynthesis of VLCFA, thereby destabilising cell membranes. Consequently, electron transport rates were reduced, resulting in lower carbon assimilation rates. Pigment content and chlorophyll fluorescence remained unaffected up to metazachlor treatments of 0.50 kg a.i. ha^{-1}, implying that photosynthesis was protected at intermediate doses of metazachlor. Surprisingly, the strong reduction of pigments in plants exposed to 0.75 kg a.i. ha^{-1}, did not affect chlorophyll fluorescence. These results indicate that the energy produced during

photosynthesis is directed to protection mechanisms of the photosynthetic apparatus, rather than to growth. This microcosm study was performed to determine how photosynthesis and growth are affected by metazachlor application on *B. napus* crop in the short-term. However, to make the transition towards the field situation, enhanced experimental set ups are required, which include natural soils. It is noteworthy that field trials have elucidated that crops exposed to pre-emergent herbicides recover from initial chemical stress and therefore it might be interesting to include additional long-term time points in future studies. To this purpose, chlorophyll fluorescence can be used to detect the occurrence of herbicide-induced stress in long-term field studies.

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Chapter 7 Case Study: Investigating the effects of metazachlor on *Brassica napus* in a long-term field experiment

7.1. Abstract

A long-term field study covering the complete life cycle of winter oilseed rape was performed to link the short-term effects of the chloroacetamide herbicide metazachlor on growth with the long-term yield and quality of the end products of the crop. Hereby, the study differentiated between the effects of biotic stress, *i.e.* weed competition, and the effects of abiotic stress, induced by metazachlor. At harvest, the reduction of crop coverage and seed yield per plant in plots without manual or chemical weed removal indicated that weed competition during the growing season inhibited plant growth, emphasizing the necessity of weed removal. Although, the advantages of chemical weed removal are manifold, metazachlor-induced abiotic stress led in the short-term to a transient reduction in crop growth when recommended doses are exceeded. However, crop plants were able to recover this initial stress during the growing season. In the long-term, inter- and intraspecific competition for light, nutrients and space, had a larger impact on crop coverage, the production of seeds and seed quality than the initial metazachlor-induced stress.

7.2. Introduction

As an oilseed crop, *Brassica napus* or rapeseed is cultivated on a worldwide scale, with Europe and Asia as its main producers (FAOSTAT 2014). The applications of its end products are versatile. As such, seed-extracted oil is either consumed as vegetable oil for human consumption or processed into biofuel. The remaining press cake serves as an additive for feed production and the remaining non-edible plant parts are used as straw for cattle or as soil amendment. *Brassica* species have been reported to possess anti-carcinogenic properties, which were attributed to the presence of compounds that are able to prevent mutagenesis and stimulate detoxification (Beecher 1994). Rapeseed-derived oil is considered a high nutritional food and feed product because of its high content of polyunsaturated fatty acids and its favourable lipid composition (Gui *et al.* 2008). The presence of linoleic (18:2) and linolenic (18:3) acids, which are respective members of the essential omega-6 and omega-3 fatty acid groups, is favourable in the mammalian diet because of their ability to reduce the risk of a heart disease and reduce cholesterol (Iggman *et al.* 2011, Lin *et al.* 2013). Additionally, oil and press cake contain proteins, minerals and vitamins,

which contribute to health by their contribution in important metabolic processes, such as lipid and protein synthesis and antioxidative defence. In addition, the quality of the oil is also represented by the content of flavonoids. Flavonoids possess a strong antioxidant capacity and contain an anti-carcinogenic power (Beecher 1994, Le Marchand 2002, Galati and O'Brien 2004). Different cultivation methods have been shown to influence the flavonoid content in *Brassica oleracea*, with higher total flavonoid contents in crop leaves grown under organic practices than when grown under conventional practices (Ferreres *et al.* 2005). Fatty acid composition in adipose tissue of rats have been found to be influenced by diets originating from different cultivation strategies (Lauridsen *et al.* 2008). As such, rats fed with vegetables and rapeseed oil from cultivations using pesticide input had higher proportions of oleic acid (18:1) and a lower proportion of linoleic acid (18:2) in adipose tissue, than rats fed with food originating from organic cultivation. Since the susceptibility towards fat oxidation increases with the level of unsaturated fatty acids, a higher proportions of saturated fatty acids to polyunsaturated fatty acids would lead to lower concentrations of liver hydroperoxides.

The increasing need for food implies the use of advanced technologies in agriculture, such as pesticide use. Hence, oil crops such as *Brassica* are being subjected to herbicide application. Metazachlor is a chloroacetamide herbicide, known to interfere with long chain fatty acid biosynthesis (Fuerst 1987, Böger *et al.* 2000). This active ingredient is applied at an early time point in the growing season: either pre-emergently, before seedling emergence, or at an early growth stage, before seedlings reach the growth stage of nine leaves (GS19) (Fytoweb). An earlier small-scale mesocosm study has shown a delay in crop emergence and settlement in the short-term under the influence of metazachlor (Chapter 3). Nevertheless, this mesocosm experiment did not reveal any differences in yield, nor in oil quality in the long-term setup. In the current study, a larger-scaled field trial was performed (i) to test the reliability of extrapolation of small-scale results to field-scale experiments, and (ii) to test the impact of pre-emergently applied metazachlor on vegetative and regenerative endpoints at harvest and seed quality, which is the source for oil and press cake applications.

7.3. Experimental design

A field trial was set-up at the experimental study site of the Agricultural University of Plovdiv in Bulgaria. The soil was a fluvisol, containing 3.7% organic matter and pH of 7.0-7.2. *Brassica napus* (cultivar Remy) seeds were sown at the end of August 2014 at 1 cm depth on plots treated with 0, 1.00, 1.25 and 2.00 kg active ingredient per hectare (kg a.i. ha^{-1}) the day after sowing. The rows were separated 12.5 cm from each other. A distinction was made between control plots with manual weed removal (0) and without manual weed removal (0+). For each condition, five replicate plots of 16 m^2 were set up according to a randomised block design. Plants were grown under rainfed conditions. Weeds were removed three times per vegetation period (*i.e.* before and after winter) in half of the control plots. Plant growth was measured two months after metazachlor application and at the end of the growing season (mid-June 2015) by measuring fresh weight of the aboveground plant parts. At harvest, vegetative and regenerative endpoints were recorded. Vegetative endpoints included plant weight, crop density and central stem height. Regenerative endpoints included the amount of branches, the number of fruits per central stem, the weight of the ten largest fruits per central stem and seed yield. After seed harvest, oil quality parameters, such as fatty acids, flavonoids and minerals were measured as described in Chapter 2 (§ 2.11 – § 2.13). Due to unexpected rainfall and incidental run-off, some replicate plots were excluded from analyses, resulting in lower biological replicates per condition (Figure 7.1).

7.4. Results and discussion

7.4.1. *Short- and long-term effects of manual weed removal*

The repeated manual removal of weeds during each vegetative growth period, *i.e.* before and after winter, resulted in a crop density of 26 plants m^{-2} and a biomass of 3.9 tonnes ha^{-1} at harvest (Figure 7.1, Supplementary file A). Without manual weeding, crop density and biomass were 24% and 25% lower than with manual weed removal, respectively. This indicated that the presence of weeds negatively influenced the emergence and settlement of crop plants during the first vegetative growing period, *i.e.* before winter, and the growth of the crop plants during the second, vegetative growing season, *i.e.* after winter (Figure 7.1). This observation implied that competition for light, water and

nutrients between crop plants and weeds interfered with the settlement and growth of the crop. Height of *B. napus* plants was similar in both absence and presence of weeds, ranging from 123 to 126 cm, respectively (Figure 7.1, Supplementary file A). Regenerative parameters were not influenced by the presence of weeds (Figure 7.1). The number of branches and fruits per central stem were similar, ranging between 10-11 branches and 49-52 fruits in both control conditions, respectively. As a result, seed production was equivalent in both control conditions, ranging between 14.6 to 15.7 g seeds per individual plant. Whereas regenerative endpoints of individual plants were not influenced by interspecific competition, the inhibition of vegetative development, such as biomass production and crop density, ultimately resulted in lower seed yield and raw material per surface area. Based on crop density and seed yield per plant, estimations about seed yield per hectare could be calculated (Supplementary file A). The absence of manual weed removal, however, lead to an estimated decrease of 30% in seed yield per hectare, with 2.83 tonnes seeds ha^{-1} in comparison with 4.02 tonnes seeds per hectare (Supplementary file A). This observation underscored the importance of weed removal.

7.4.2. *Does the presence of weeds alter the quality of the seeds?*

The quality of the oil and the crude extract were considered by means of fatty acid composition, flavonoid profile and mineral content in seeds. The presence of interspecific competition during the growing season ultimately resulted in a change in flavonoid and mineral composition (Tables 7.1 and 7.2). As such, kaempferol-glucoside content was 17% lower in oil derived from plants grown on plots without manual weed removal (0.079 µmol g^{-1}) in comparison with oil derived from plants grown on plots with manual weed removal (0.095 µmol g^{-1}) (Table 7.1). The levels of phosphorus (P) and potassium (K) in seeds derived from plants grown in plots containing weeds were 9.0 and 4.3 mg g^{-1}, respectively, and were both 8% lower than the levels in seeds derived from plants grown on manually weeded plots, with 9.8 and 4.7 mg g^{-1} seeds, respectively (Table 7.2).

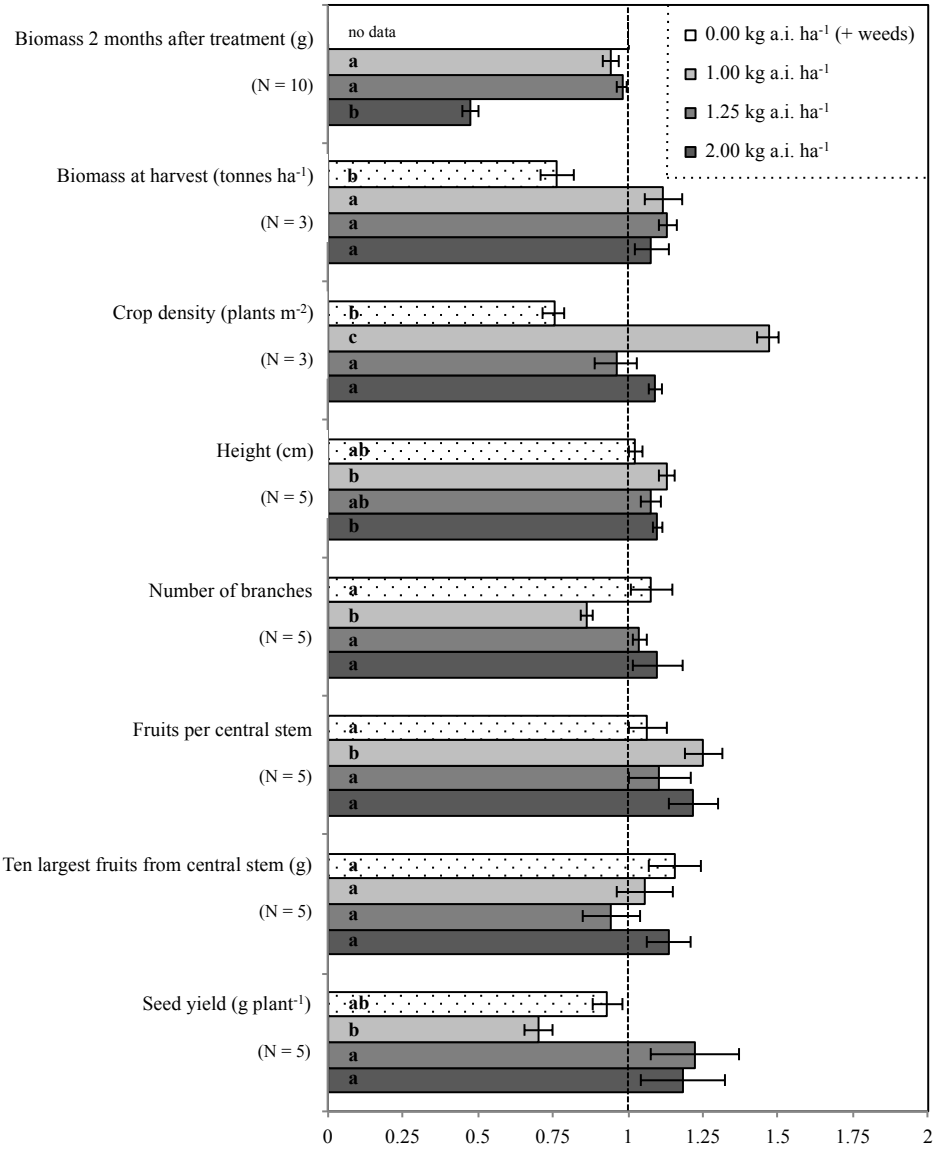

Figure 7.1. *Vegetative and regenerative endpoints of* B. napus *exposed to 0 kg (dotted), 1 kg (light grey), 1.25 kg (grey) and 2 kg (dark grey) metazachlor ha^{-1}, expressed as relative values. The dotted line represents the control plots with manual weed removal and is set as reference value. Results are represented as relative mean values of minimal three biological replicates ± SE. Different letters represent significant differences, with 'a' representing no difference with the control line. Post-hoc values: $p < 0.05$. Absolute values are represented in supplemental file A.*

7.4.3. *Short- and long-term effects of chemical weed removal*

Two months after the pre-emergent application of the herbicide metazachlor, the growth of *B. napus* was not impaired by metazachlor doses up to 1.25 kg a.i. ha^{-1}, since weight of the aboveground plant parts was approximately 2 g per individual plant for all treatments (Figure 7.1, Supplemental file A). This result points out that the pre-emergent application of the recommended dose of 1.0 kg a.i. ha^{-1} metazachlor had no influence on crop growth within two months after application. Nevertheless, it remains unclear how plants have developed before this time point and whether treated plants had recovered from initial herbicide induced toxicity. Indeed, in an analogous mesocosm experiment it was revealed that one month after application of 1.25 kg a.i. ha^{-1} the growth of *B. napus* was reduced (Chapter 3). Nevertheless, the result of the current study was in line with results obtained in an analogous microcosm experiment in which nine-weeks-old *B. napus* plants recovered from the initial metazachlor-induced reduction of growth with regard to the biomass of aboveground plant parts (Chapter 4). Hence, it can be stated that the probable occurrence of initial metazachlor-induced toxicity and succeeding recovery, in terms of growth, happened within two months after application. Exposure towards a two-fold dose of metazachlor, resulted in a 52% reduction of plant weight, with an average plant weight of 1 g (Figure 7.1). This observation emphasizes the reliability and the importance of the recommended application dose of the active ingredient.

At harvest, biomass production of *B. napus* was equal in all treatments, resulting in yields within a range of 3.9 to 4.4 tonnes ha^{-1}. With exception of the lowest metazachlor treatment (1.0 kg a.i. ha^{-1}), plant density was equal in all treatments with on average 26 to 28 plants m^{-2} at the end of the growing season (Figure 7.1, Supplementary file A). Remarkably, crop coverage was highest in plots treated with the recommended dose of 1.0 kg a.i. ha^{-1} with 38 plants m^{-2}, which was 1.47 times higher than in control plots. This result was interesting, because it supported the assumption that pre-emergent application dose of 1.0 kg a.i. ha^{-1} inhibited weed and *B. napus* seedling emergence and settlement in the short-term in such degree that it resulted in a maximal seedling survival. After all, it is estimated that 35 plants m^{-2} is an optimal

density for most *B. napus* varieties (BASF). The results indicated that higher doses of metazachlor initially inhibited in the settlement of weeds and crop plants.

Vertical growth (in terms of height) was 1.13 and 1.1 times higher in 1.0 and 2.0 kg a.i. ha^{-1} plots, in comparison with manually weeded control plots, respectively (Figure 7.1). On the one hand, this observation could be explained by the absence of the occasional presence of weed competition that takes place in control plots that are manually weeded. In fact, manual weeding is less efficient in prevention of weed growth because different weed species emerge from the seed bank in the soil at specific times of the year. Since weeds can only be removed after they have been established, short periods of weed presence in manually controlled plots can account for inhibition of stem growth during the vegetative period. Moreover, the manual weed removal can also induce mechanical disturbance. Another explanation for the higher stem height under 1.0 kg a.i. ha^{-1} treatment could be related to the presence of intraspecific competition, whereby the competition for light between individual crop plants stimulates vertical growth. The higher stems of plants treated with 2.0 kg a.i. ha^{-1} could be the result of an increased development due to high exposure in terms of survival.

Regarding the regenerative characteristics, it can be stated that with exception of 1.0 kg a.i. ha^{-1} treatment, all treatments resulted in 10 to 11 branches per plant, 49 to 61 fruits per central stem and 2.7 to 3.0 g of ten largest fruits from the central stem at harvest, which finally resulted in a seed yield of 16 to 19 g per individual plant (Figure 7.1, Supplementary file A). It was remarkable that the pre-emergent application of the recommended dose resulted in significantly lower amounts of branches per plant (mean of 8.8) in comparison with non-treated plants (mean of 10.2), leading to a lower mean seed yield per plant of 11 g per plant in comparison with non-treated plants that yielded 16 g per plant (Figure 7.1, Supplementary file A). The deviating regenerative characteristics of plants exposed to 1.0 kg a.i. ha^{-1}, could be explained by the higher crop density, which was accompanied by a higher degree of intraspecific competition during the growth period.

Table 7.1. *Fatty acid and flavonoid profiles of seeds harvested from different cultivations; 0, 1.0, 1.25 and 2.0 kg metazachlor per hectare. A difference was made between control plots with (0+) and without weeds (0). Due to lack of replicates for fatty acid and flavonoid profiles, differences are highlighted in case of more than 5% (light grey), 10% (grey) and 20% (dark grey) difference with the control value.*

Seed quality parameters	Unit	Metazachlor (kg a.i. ha^{-1})				
		0	0+	1.0	1.25	2.0
Fatty acid profile						
C16:0 (Palmitic acid)	%	4.71	4.79	**4.40**	4.66	4.75
C16:1 (Palmitoleic acid)	%	0.25	**0.26**	**0.23**	0.24	0.25
C18:0 (Stearic acid)	%	1.65	1.73	1.67	1.71	1.61
C18:1 (Oleic acid)	%	62.25	62.89	63.82	63.32	62.34
C18:2 (Linoleic acid)	%	21.05	20.37	**19.90**	20.40	21.04
C18:3 (α-linolenic acid)	%	8.18	8.08	8.01	7.85	8.16
C20:0 (Arachidic acid)	%	0.50	**0.53**	0.51	0.50	**0.47**
C20:1	%	1.08	1.11	1.09	1.08	1.07
C20:2	%	0.07	0.00	0.07	0.00	0.07
C22:0 (Behenic acid)	%	0.25	0.24	0.24	0.24	0.24
C22:1 (Erucic acid)	%	0.00	0.00	0.05	0.00	0.00
C22:2	%	0.00	0.00	0.00	0.00	0.00
C18:2 / C18:3	ratio	2.57	2.52	2.48	2.60	2.58
Flavonoid profile						
Total	µmol g^{-1}	2.518	2.491	**2.279**	**2.814**	2.549
Kaempferol-glucoside	µmol g^{-1}	0.095	**0.079**	**0.077**	**0.114**	0.091
Kaempferol-3-O-sinapoyl-sophoroside-7-glucoside	µmol g^{-1}	2.378	2.368	**2.172**	**2.648**	2.401
Quercetin-3-O-sophoroside	µmol g^{-1}	0.021	0.021	**0.012**	**0.013**	**0.017**
Kaempferol-3-O-caffeoyl-sophoroside-7-O-glucoside	µmol g^{-1}	0.025	**0.023**	**0.018**	**0.038**	**0.039**

7.4.4. *Does the application of herbicides affect the quality of the seeds?*

The results concerning the quality of seeds derived from plants exposed to pre-emergent application of metazachlor indicated shifts in the fatty acid, flavonoid and mineral composition in comparison with seeds originating from plants grown on manually weeded plots. Seeds harvested from plants that received 1.0 kg a.i. ha^{-1} treatment, contained 6.6% and 5.5% lower levels of palmitic acid (C16:0) and linoleic acid (C18:2), respectively, and a 2.5% higher level of oleic acid (C18:1) in comparison with control seeds-derived oil (Table 7.1). These results suggest a higher conversion rate of palmitic acid into oleic acid and a lower desaturation of oleic acid into linoleic acid. This observation suggests a causal link between the presence of metazachlor and the lower amount of polyunsaturated fatty acids. This can be explained by the working mechanism of metazachlor as it inhibits fatty acid desaturases (Weisshaar *et al.* 1988, Möller and Albrecht 1994). However, an analogous field study has pointed out that metazachlor was completely metabolised in aboveground plant parts of *B. napus* 88 days after treatment (Personal communication). Therefore, it is less likely that the observed alterations in fatty acid profile in seeds are due to direct action of the active ingredient. Moreover, the exposure to higher amounts of metazachlor does not induce similar alterations, indicating that the presence of high plant coverage and thus intraspecific competition is more likely to underlie these changes in lipid profile. Ijaz and Honermeier (2012) ademonstrated that also the application of fungicidal compounds could suppress linoleic acid synthesis in favour of oleic acid in winter rapeseed oil. Vegetable oils are an important source of unsaturated fatty acids for human nutrition, being a source of energy, vitamin E, essential polyunsaturated fatty acids and phenolic compounds, such as flavonoids (FAO 2010). A positive correlation was found between the presence of vitamin E and the degree of unsaturation in vegetable oil (Kamal-Eldin and Andersson 1997). Fatty acid profile of vegetable oil in general is mainly composed from the monounsaturated fatty acid, oleic acid (18:1) (≈ 60%), linoleic acid (≈ 20%), linolenic acid (≈ 10%) and saturated fatty acids (≈ 10%) (Dubois *et al.* 2008). Fatty acid profiles from seeds of all treatments corresponded with the general profile of vegetable oils (Table 7.1).

Table 7.2. *Mineral profile of oil extracted from seeds harvested from different cultivations; 0, 1.0, 1.25 and 2.0 kg metazachlor per hectare. A difference is made between control plots with (0+) and without weeds (0). Data are represented as average values of three biological replicates ± SE. Values significantly different from control value (0) are represented in bold and grey (Tukey post-hoc p < 0.05).*

Seed quality parameter	Unit	Metazachlor (kg a.i. ha^{-1})				
		0	0+	1.0	1.25	2.0
Mineral profile of seeds						
Macronutrients						
P	(mg g^{-1} seed)	9.8 ± 0.1	**9.0 ± 0.05**	**8.0 ± 0.1**	**8.5 ± 0.05**	**9.1 ± 0.1**
K	(mg g^{-1} seed)	4.7 ± 0.03	**4.3 ± 0.02**	**4.3 ± 0.1**	4.4 ± 0.03	4.6 ± 0.1
Ca	(mg g^{-1} seed)	4.3 ± 0.1	4.1 ± 0.1	4.5 ± 0.02	4.4 ± 0.1	4.4 ± 0.1
S	(mg g^{-1} seed)	3.7 ± 0.1	3.8 ± 0.4	3.9 ± 0.1	3.8 ± 0.1	3.7 ± 0.03
Mg	(mg g^{-1} seed)	2.8 ± 0.02	2.9 ± 0.01	2.8 ± 0.05	2.9 ± 0.02	2.8 ± 0.03
Micronutrients						
Fe	(mg g^{-1} seed)	49 ± 0.6	54 ± 1.0	**55 ± 2.5**	51 ± 0.5	53 ± 0.6
Zn	(mg g^{-1} seed)	36 ± 0.2	**45 ± 0.4**	**42 ± 0.03**	**38 ± 0.8**	**40 ± 0.4**
Mn	(mg g^{-1} seed)	35.7 ± 1	38.4 ± 1	36.2 ± 0.1	38.0 ± 0.7	37.9 ± 0.9
Cu	(mg g^{-1} seed)	18 ± 0.02	18 ± 0.1	**21 ± 0.2**	18 ± 0.2	18 ± 0.1
Na	(mg g^{-1} seed)	9.0 ± 0.5	5.7 ± 0.4	9.9 ± 1.2	8.8 ± 1.4	6.6 ± 0.2

A balanced amount of linoleic and linolenic acids within the human diet can impact health as they control cardiovascular risk, blood lipids and blood cholesterol (EFSA 2010). High ratios of omega 6 to omega 3 lipids, which are present in the Western diet, have been correlated with the risk of cancer and chronic vascular, inflammatory and autoimmune diseases (Simopoulos 2002). In addition, flavonoids are known to act as natural antioxidants and have also been associated with a decreased risk of the above-mentioned diseases (Spencer *et al.* 2008). Due to lack of replicate measurements, solid statements about flavonoid content in the seeds could not be made. Quercetin seemed to be the most sensitive flavonoid to metazachlor, as it was lower in all seeds derived from plants grown on metazachlor-treated plots (Table 7.1). With exception of quercetin, flavonoid content tended to be equal or higher in seeds derived from plants grown on plots treated with 1.25 and 2.0 kg a.i. ha^{-1} metazachlor (Table 7.1). However, it was apparent that the levels of each individual flavonoid

in seeds derived from plants grown on plots receiving 1.0 kg a.i. ha^{-1} metazachlor were lower than in seeds derived from plants grown on manually weeded plots, resulting in a reduction of 9.5% of total flavonoid content in comparison with control seeds (Table 7.1). The lower flavonoid content under influence of the recommended metazachlor dose (1.0 kg a.i. ha^{-1}) could probably be related to the presence of intraspecific competition. Concerning the mineral content of the seeds, phosphorus content was 7.2 - 18.4% lower in seeds derived from plants grown on plots treated with 1.0 to 2.0 kg a.i. ha^{-1} metazachlor, although not in a dose-dependent way. The mineral profile of seeds derived from plots treated with the recommended plots was most different from control seeds, with lower phosphorus and potassium contents and higher levels of copper, iron and zinc.

In conclusion, the results of this study reveal that chemical weed control with metazachlor in winter oilseed rape cultivations, is essential and that is has a dual action. Firstly, the active ingredient excludes initial biotic stress from interspecific competition by the prevention of weed settlement. Secondly, its phytotoxic effect on the crop plant results in an optimal crop density that enables the emerged crop plants to develop and grow at an optimal area whereby intraspecific competition is minimal and interspecific competition is excluded. Regarding the quality of the oil, no clear changes were observed under the influence of metazachlor. However, extra oil parameters such as total antioxidative capacity of the oil and press cake should be included in future studies, along with the inclusion of a greater amount of biological replicates.

7.5. References

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7.6. Supplementary files

Supplementary file A. *Absolute values of vegetative and regenerative endpoints of* B. napus*, grown on field plots pre-emergently treated with 0, 1.0, 1.25 and 2.0 kg metazachlor per hectare (kg a.i. ha^{-1}). A difference is made between control with and without weeds, with 0+ control plots with weeds.*

		Metazachlor (kg a.i. ha^{-1})				
Parameter	**Unit**	**0**	**0+**	**1.0**	**1.25**	**2.0**
Short-term						
Aboveground weight	(g plant^{-1})	2.11 ± 0.03	2.00 ± 0.03	2.00 ± 0.06	2.07 ± 0.04	**1.01 ± 0.04**
Long-term (harvest)						
Biomass	(tonnes ha^{-1})	3.9 ± 0.4	**3.0 ± 0.2**	4.3 ± 0.2	4.4 ± 0.1	4.2 ± 0.2
Crop density	(plants m^{-2})	26 ± 1	**19 ± 1**	**38 ± 1**	25 ± 2	28 ± 1
Height	(cm)	123 ± 2	126 ± 3	**139 ± 3**	133 ± 4	**135 ± 2**
Number of branches		10.2 ± 1.0	11.0 ± 0.7	**8.8 ± 0.2**	10.6 ± 0.2	11.2 ± 0.9
Fruits per central stem		49 ± 2	52 ± 3	**62 ± 3**	54 ± 5	60 ± 4
Weight of 10 largest fruits from central stem	(g)	2.7 ± 0.3	3.1 ± 0.2	2.9 ± 0.3	2.6 ± 0.3	3.1 ± 0.2
Seed yield	(g plant^{-1})	16 ± 1	15 ± 1	**11 ± 1**	19 ± 2	19 ± 2
Seed yield	(tonnes ha^{-1})	4.02 ± 0.14	**2.83 ± 0.13**	4.15 ± 0.10	4.73 ± 0.34	**5.19 ± 0.11**

Chapter 8 General discussion and future perspectives

8.1. How does *Brassica napus* cope with metazachlor application?

8.1.1. Approach

In a world relying on agrochemical application for food production it is important to monitor the fate of the applied compounds in the environment as well as their effect on the crops harvested on fields after application of such chemicals. With the conduction of the presented studies (Chapters 3-7) we have attempted to demonstrate the effects of the pre-emergence herbicide, metazachlor, on one variety of winter oilseed rape (*Brassica napus*, var. Remy), which is commonly used as food crop for oil and high-protein press cake, as well as biofuel crop. Recently, winter oilseed rape has also been proposed to function as a possible phytoextracting crop suitable for phytoremediation purposes (Thewys *et al.* 2010, Van Slyken *et al.* 2013). The choice of *B. napus* as test organism was based on its significance in several domains of agriculture on the one hand and in upgrading low-valuable, contaminated soils in phytoremediation practices on the other hand. In addition, there is an extensive know-how of the research team concerning the genetically related model plant *Arabidopsis thaliana*. Metazachlor was used as a herbicide as it is often used in *Brassica* cultivations. This compound belongs to the chloroacetamide class of herbicides, and as such it is assumed to impair the formation of long chain fatty acids. This in its turn could imply consequences regarding the quality of the oil.

Different experimental set-ups allowed us to investigate the effects of metazachlor at different time points in the life cycle of the crop and at different biological organisation levels. In addition, the different experimental designs enabled to link the occurrence of herbicide-induced phytotoxicity in the short-term to processes of detoxification and repair. Furthermore, the link between short-term observations and long-term vegetative and regenerative development and quality of the seeds could be investigated. Mesocosm and microcosm experiments aimed at determining the occurrence and the degree of herbicide-induced toxicity in the crop. In the microcosm experiments the focus was set on the underlying cellular processes determining crop tolerance against the herbicide in the short-term. Long-term mesocosm and field experiments could link the short-term herbicide-induced effects to long-term vegetative and

regenerative development and quality of the seeds. Microcosm and mesocosm test systems are limited in size and duration of the experiment, and they have boundaries that restrict interaction with the rest of the ecosystem. Nevertheless they are considered as useful tools to test single-species toxicity as well as agricultural ecosystems (Van den brink *et al.* 2005).

8.1.2. Short-term phytotoxicity under influence of pre-emergent application of metazachlor

As it is most common in agricultural practice, metazachlor is applied one day after sowing of the seeds. Both microcosm and mesocosm experiments revealed that, within one month after application, the pre-emergent application of metazachlor was phytotoxic towards *B. napus*. Metazachlor-induced phytotoxic effects were present in *B. napus* at different biological organisation levels. Although the germination of seeds was not affected, mortality rates of young seedlings were situated between 12.5% and 20% three weeks after treatment with metazachlor. This observation indicates that the survival of young seedlings was impaired, even at recommended application doses. From the moment leaves appeared, morphological deviations were displayed as crinkled leaves, shortened mid ribs and incompletely detached leaves. Growth, in terms of stem height and aboveground weight, was inhibited by metazachlor (Figure 8.1). Results from the microcosm set-up revealed that metazachlor-induced phytotoxicity also occurred at the cellular level, in the form of lipid peroxidation of membranes. Membrane damage could be addressed to either the direct action of metazachlor or through the action of metazachlor-induced reactive oxygen species (ROS) (Figure 8.2).

Considering the rate of leaf development, which was solely monitored in the microcosm set-up, no effect of metazachlor up to seven weeks after treatment was observed. However, between five and seven weeks after treatment leaf development occurred at a slightly higher rate in herbicide-exposed plants. Considering the growth of the aboveground plant parts, the initial differences in weight disappeared within nine weeks after treatment. This observation was confirmed by results obtained from the field experiment, which showed that the

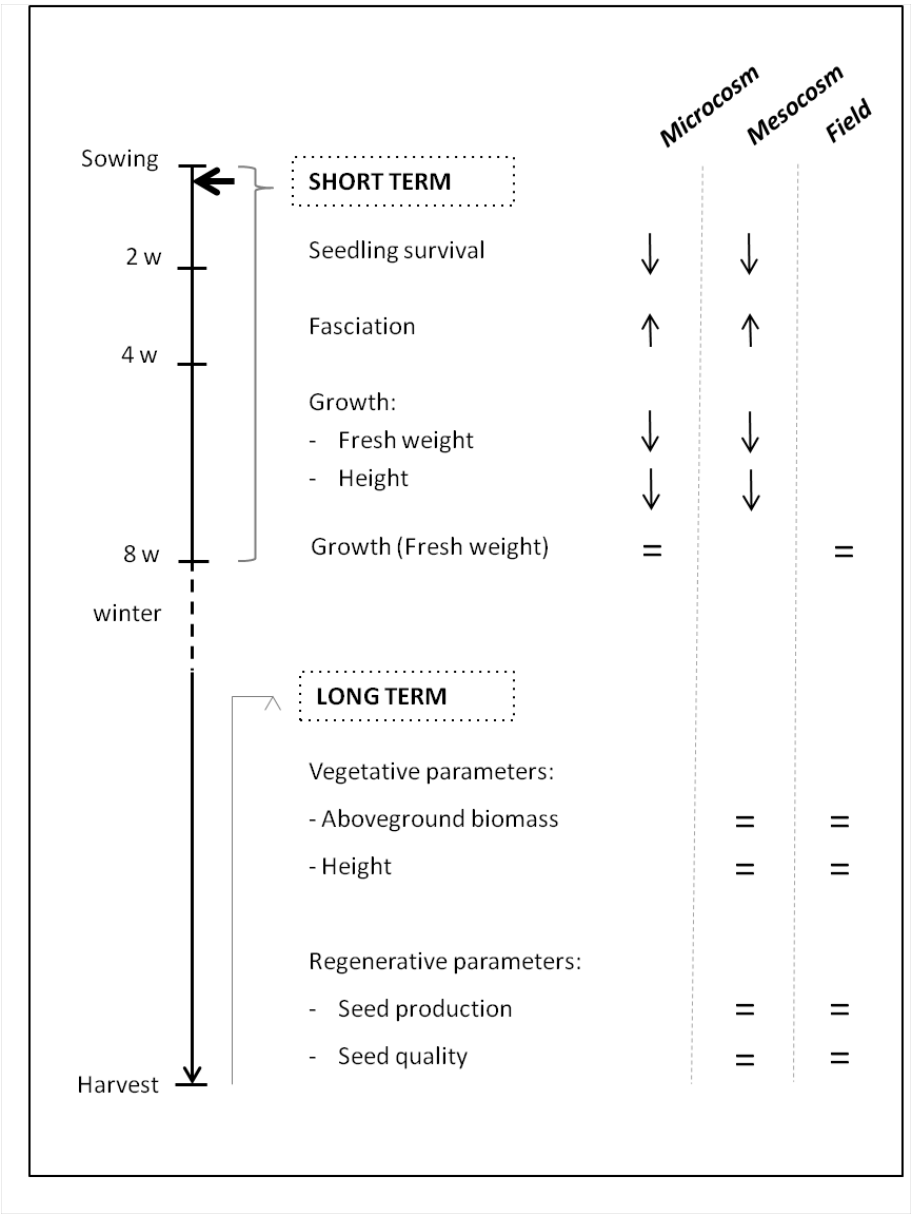

Figure 8.1. *Overview of the growth of* B. napus*, when subjected to pre-emergent application of metazachlor. Time points during its development and the respective measurements and results are given per experimental set-up.*

biomass of *B. napus* was not affected by the recommended metazachlor doses, two months after application.

8.1.3. Mechanisms underlying the ability of *Brassica napus* to recover

In order to get more insight into the underlying components and mechanisms behind herbicide tolerance and recovery in crops, controlled laboratory experiments were conducted in microcosms (Chapters 4 and 5). Results obtained from the microcosm studies revealed that metazachlor is actively being neutralised by the crop, within four weeks after application. Firstly, the internal metazachlor concentration in the aboveground plant parts between two and four weeks after treatment decreased. Secondly, a two-fold increase in GST activity towards different substrates 1-chloro-2,4-dinitrobenzene (CDNB), fluorodifen, p-nitrophenylacetaat (p-Npa) and a two-fold decline in glutathione (GSH) content at this time point were observed. Taken together, these observations support the hypothesis that phase II of the detoxicifation metabolism is activated whereby metazachlor is actively being metabolised and conjugated to GSH by GST. Since the exposure to metazachlor resulted in the inhibition of growth within two weeks after treatment, a closer look was taken at detoxification at this time point too. Early activation of GST activity (towards CDNB and Npa) and a shift in GSH redox state toward its oxidised form indicated that the detoxification metabolism was already active two weeks after treatment.

The occurrence of membrane damage, in the form of increased membrane lipid peroxidation and leakage of potassium, indicated the presence of ROS and hence metazachlor-induced oxidative stress. Oxidative stress can cause structural damage to DNA, lipids, proteins and enzymes within the cell. Therefore, plant cells are provided with an antioxidant defence network, comprising antioxidative enzymes and metabolites. Maintenance of the cellular redox state is important in a plant's ability to withstand stress. The increased activity of antioxidative enzymes (SOD, CAT, GR and GPx) and the increased capacity of the lipophilic fraction of antioxidants, two and four weeks after metazachlor treatment, respectively, indicated that the antioxidative response was activated in order to detoxify metazachlor-induced ROS.

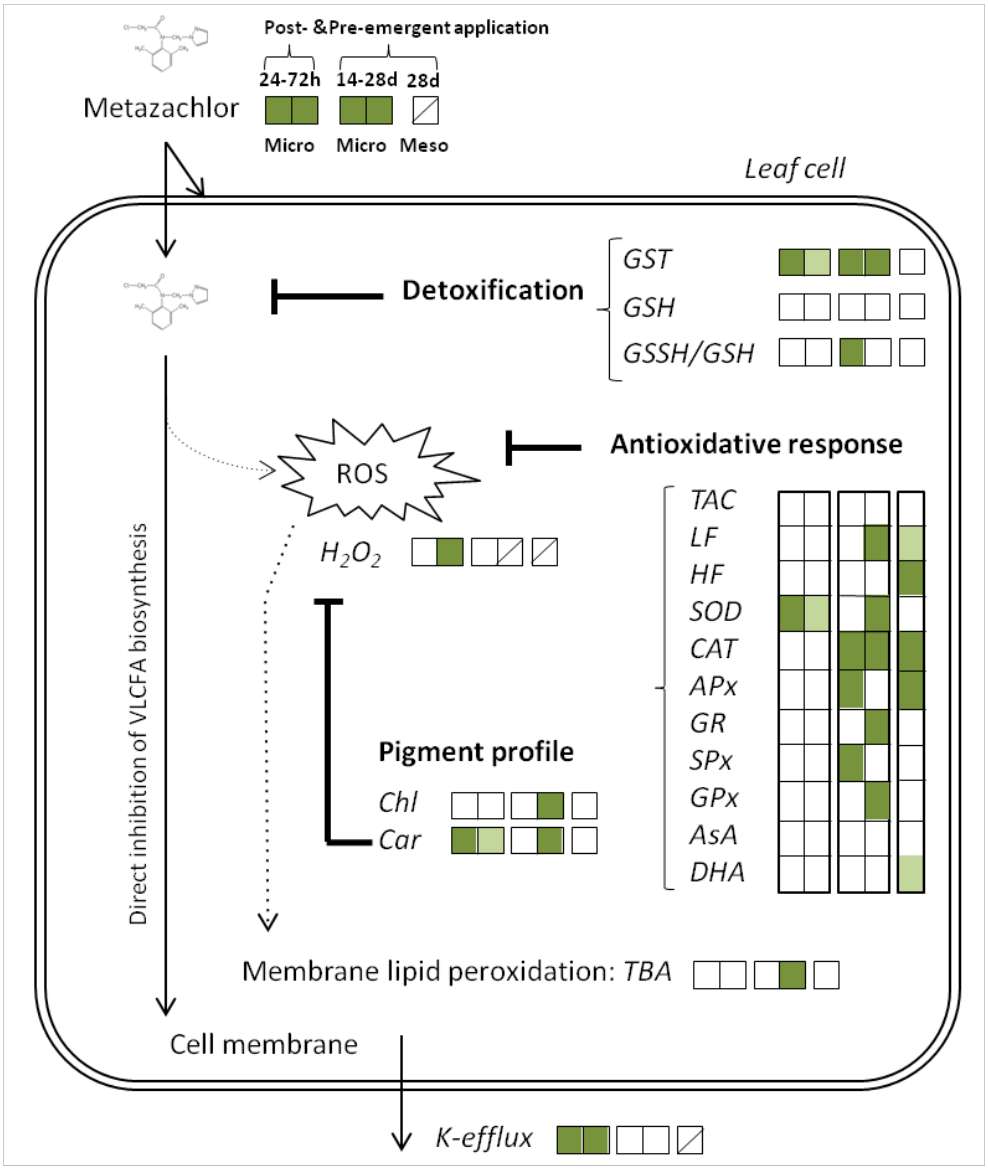

Figure 8.2. *Overview of the cellular changes induced by metazachlor on leaf cells of* B. napus*. Metazachlor induces direct (solid line) disturbance of cell membrane integrity, thereby causing K leakage, and indirect (dotted line), via the production of ROS, thereby inducing membrane lipid peroxidation. Components involved in the detoxification metabolism and the antioxidative response are represented in italic. Increments and reductions in concentration or in activity of each component are represented in dark green and light green, respectively. Cubes with a diagonal indicate that the component was not measured at the respective time point. The cubes represent from left to right; 24 HAT (post-emergent application), 72 HAT (post-emergent application), 14 DAT (pre-emergent application), 28 DAT (pre-emergent application) and 28 DAT (pre-emergent application in mesocosm).*

It was interesting to notice that photosynthesis remained unaffected despite the strong reductions in pigment concentrations after exposure to higher than recommended metazachlor concentrations (Chapter 6). These results suggest that the process of photosynthesis was protected even at high exposure concentrations and at low chlorophyll and carotenoid pigment concentrations. Serra *et al.* (2015) have found similar results, whereby the efficiency of PS II remained unaffected after thirty-day exposure of *Italian ryegrass* to glyphosate, its degradation product aminomethylphosphonic acid (AMPA) and the fungicide tebuconazol, despite a reduction of pigments. Linking the phytotoxic effects at morphological level with the cellular processes in the plant leaves, the results indicate that the chemical energy, derived from photosynthesis, was consumed by defence mechanisms rather than by the growth metabolism. Hence, energy was consumed by processes involved in the protection of the photosynthetic apparatus, the detoxification of the xenobiotic compound and the maintenance of the cellular redox state. The re-allocation of energy from biomass production into the development of leaves and the biosynthesis of photosynthetic pigments could be a successful strategy to survive, leading to a faster development of reproductive organs, thereby increasing their fitness (Sulmon *et al.* 2015).

8.1.4. At what rate are underlying mechanisms of tolerance activated?

To get an idea about the acute effects of metazachlor on the crop, a microcosm experiment was set-up whereby metazachlor was applied in a post-emergent manner on 14-day-old plants (Chapter 5). The increased cellular potassium efflux indicated that metazachlor was able to inhibit very long chain fatty acid synthesis within 24h after treatment. The simultaneous increment in GST and SOD activity, indicated the early activation of both metazachlor detoxification and antioxidant defence. The absence of membrane lipid peroxidation at this time point revealed that both processes are effective in preventing cellular damage induced by ROS. However, 72 h after treatment the increased levels of internal metazachlor induced an oxidative burst in leaf cells, which was accompanied by a decrease in GST and SOD activity. Hence, it can be stated that at this time point young seedlings were very vulnerable to metazachlor. This was reflected in a an immediate stagnation of shoot and root growth and a temporary delay in leaf development and a lower aboveground weight, seven

weeks after treatment. Although the underlying mechanisms involved in herbicide detoxification and maintenance of the cellular redox state are rapidly activated in leaf cells, i.e. within 24 hours after treatment, these processes were inhibited by metazachlor 72 hours after treatment. Hence, the dual of ROS in signalling versus damage should be further investigated.

8.1.5. Recovery in the long-term

A preliminary mesocosm experiment (Chapter 3) revealed an indirect positive side effect of initial chemical stress on the density of the crop plants. In the absence of metazachlor and weeds, intraspecific competition between crop plants led to a reduction in biomass and seed production at harvest. Because metazachlor inhibits seedling settlement, the ultimate amount of crop plants that complete their life cycle and produce seeds was lower under influence of metazachlor. As such, biotic competition for light, nutrients and space is minimalized. Hence, growth and seed production was assured, emphasizing the importance of chemical weed control.

Excluding intraspecific competition in control plots, the results gathered from both long-term set-ups, in mesocosms (Chapter 3) and at the field (Chapter 7), indicate that metazachlor is not affecting the aboveground biomass nor the quality of *B. napus*' end products. As such, fatty acid profile, flavonoid profile and nutrient profiles of seeds were not affected by pre-emergent metazachlor application. Overall, results derived from both mesocosm and field set-ups were analogues, pointing out that *B. napus* is able to recover from initial induced chemical stress and that small-scale mesocosms can be representative test systems for testing the effect of chemicals in crops.

8.2. Implications of this study for agriculture and science

According to the current application conditions, metazachlor can ensure the quality of the end products of oilseed rape for different markets. Remarkably, application doses that were twice the recommended dose did not induce any changes in flavonoid, fatty acid and nutrient profile of the seeds, nor aboveground biomass production. However, more quality parameters, such as total antioxidative capacity (TAC) and peroxide value of the oil, can be included in future studies to validate this statement. Winter oilseed rape is cultivated for

consumption in food and biofuel industry and is also known to be an accumulator of heavy metals, useful in phytoremediation practices (Meers *et al.* 2010, Croes *et al.* 2013). The outcome of this study is crucial for end products destined for food industry. Winter oilseed rape is mainly used for this purpose. With more advanced techniques of second generation biofuels, where also non-edible, high cellulose-containing parts can be transformed into biofuels (EASEC 2012), oilseed rape will become more interesting as biofuel crop. Taking into account the current food vs. feed debate, marginal soils can serve to cultivate oilseed rape for biofuel purposes. Addition of selected growth-promoting bacteria and soil amendments in the form of e.g. biochar can stimulate this opportunity (Cornelissen *et al.* 2005, Dechene *et al.* 2014).

Although the intensification of agronomic practices is promising, this also harbours significant constraints. Along with the increased cultivation of pesticide-intensive crops, such as oilseed rape, especially when grown in monocultures or on formerly set-aside land or converted grassland, risks are associated. Over-usage of fertilisers and pest control poses serious threats to surrounding terrestrial ecosystems and downstream aquatic ecosystems (Loos *et al.* 2010). Hence, biodiversity decreases and ecosystem services are being threatened. Therefore, Bunzel and colleagues (2014) recommend including energy crops into existing food production systems. Optimised cultivation systems with diverse crop rotations could help to improve monotonous agricultural landscapes thereby increasing biodiversity and minimise pesticide exposure. In the future, integrated pest management will become more important in the context of food security without climate change and environmental implications (Tilman *et al.* 2002, Geiger *et al.* 2010). This approach combines a minimal use of chemical pest control with alternative pest control measurements, utilizing biodiversity-based ecosystem services, such as biological pest control.

Results obtained in this study could potentially contribute to herbicide development and biotechnological applications, such as the design of genetically modified herbicide-tolerant crops. Since differences in herbicide tolerance between plant species depend on the activation and the rate of detoxification,

knowledge about GST and GSH can contribute to optimisation of application doses of new herbicides in herbicide development. In addition, as biotechnology brings complementary advantages towards safeguarding the yields in the agronomic sector, identification of genes involved in securing key crop yields is the focus in herbicide development.

8.3. Future perspectives

In conclusion, the ability of *B. napus* to overcome the initial metazachlor-induced damage before winter was addressed to (i) the early activation of enzymes and metabolites involved in detoxification, for the immediate neutralisation of metazachlor, and (ii) to enzymes and metabolites involved in the maintenance of the cellular redox state, to cope with indirect metazachlor-induced oxidative stress. Whereas recovery was recorded when the herbicide was pre-emergently applied, it remains unclear if recovery occurs when metazachlor is applied during early growth stages of the crop. A long-term experiment with post-emergent application of metazachlor could reveal whether the time point of application contributes to the tolerance of a crop. In relation to end products' quality, total antioxidative capacity could be a valuable parameter, in addition to the fatty acid, flavonoid and nutrient profiles that have been monitored in this study, and could be included in agronomical studies.

Gene expression analyses might be used to explore changes at the transcript level, which is important for future biotechnological approaches. It might also be a useful tool in the search for substrate specificity of GST isoforms, which might be involved in fine-tuning specific responses to xenobiotic stress. In order to use GST as a biomarker for tolerance of crops in toxicity tests, research should focus on the delicate interaction between components of the detoxification metabolism and components of the cellular redox state to untangle the signalling versus damage function of ROS. Therefore, it would be interesting to investigate the detoxification metabolism in parallel in a related weed species *B. napus*, *i.e. Arabidopsis thaliana*, of which the whole genome is sequenced, multiple mutants are readily available and biotechnological tools are well established.

In general, comparative studies between crop and weed species towards herbicide exposure can give more insights into the differences in tolerance essential for agronomic applications. As such, the predictive value of short-term parameters, regarding detoxification and cellular redox state, can be studied in determining the outcome of crop yield and quality in the long-term in eco-toxicological studies.

Although the focus of this study was set on the impact of an agrochemical on a crop, the potential effects on the surrounding organisms and ecosystems should also be considered. To include for example soil organisms or insects that use crops for foraging or potential run-off, additional field trials and mesocosms experiments can be conducted. These experimental set ups create the possibility to monitor soil composition, to follow up species abundance and diversity in terms of biodiversity, to sample vulnerable downstream ecosystems and to determine their impact on ecosystem services.

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Scientific output

International Journals

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- **Vercampt, H., J. Vangronsveld and A. Cuypers (2015).** Detoxification of chloroacetamide herbicide metazachlor and its relation with short- and long-term trade-off in yield and quality of *Brassica napus*. 18th International Plant Protection Congress. Berlin, Germany. August 24th to 27th 2015. Abstract of oral presentation.

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