**NTNU – Trondheim**
Norwegian University of
Science and Technology

# Studying attractiveness and defense responses of Brassica napus transgenic MINELESS plants to insects for pest management

## Aysel Heydarova

Biotechnology
Submission date: September 2014
Supervisor: Atle M. Bones, IBI
Co-supervisor: Ishita Ahuja, IBI

Norwegian University of Science and Technology
Department of Biology

*Dedicated to my beloved family*

# Acknowledgements

Finally a lot of hugs to my family and friends for their support and motivation throughout all my master study.

# Abstract

"Glucosinolate–myrosinase" system is well-established defense mechanism against insect herbivores feed on plants from Brassicaceae family. This defense mechanism is also known as "the mustard oil bomb" which bring myrosinase enzyme and glucosinolate together upon insect attack and tissue disruption and cause glucosinolate hydrolysis by myrosinase enzyme activity. Glucosinolate and its hydrolysis products have a deterring effect on herbivores, and protect plant parts from insect damage. The modified plants have been named *MINELESS* due to lack of myrosin cells.

The aim of this study was to get an insight into defense responses of *MINELESS* and wild- type *Brassica napus* plants after being challenged by insects *Delia radicum*, *Delia floralis*, *Mamestra brassicae* and *Meligethes aeneus*. The hypothesis behind this work were due to lack of myrosin cells and the enzyme myrosinase, the glucosinolate-myrosinase system is modulated and *MINELESS* plants possess more glucosinolates in plant tissues. Glucosinolate have adverse and deterring effects on insect herbivores, and larvae of generalist herbivore, *Mamestra brassicae* preferred to feed on wild-type cotyledons of *Brassica napus* plants, which contain lower amounts of glucosinolates than those of *MINELESS*. Role of glucosinolates on attraction of pollen beetles, *Meligethes aeneus*, is not exactly known, however, pollen beetles are attracted by glucosinolates hydrolysis products, isothyocyanates. Wild-type plants were preferred by pollen beetles for feeding over *MINELESS* plants which possess high levels of glucosinolates, but low levels of isothiocyanates due to lack of myrosinase activity.

Four glucosinolates were detected with the highest amount of indol-3-ylmethyl- (I3M) glucosinolate both in control and insect challenged aboveground tissues, with slightly higher amounts in *MINELESS* plants than wild-type. However total glucosinolate levels did not show exact difference between *MINELESS* and wild-type plants. I3M glucosinolates levels increased upon herbivore feeding in all treatment days. Increasing days of treatment increased levels of aliphatic glucosinolate, 5-methylsulphinylpentyl- (5MSOP) glucosinolate and decreased levels of indole glucosinolate, I3M glucosinolate.

In both no-choice and free choice experiments, *M. brassicae* larvae consumed more wild-type cotyledons than those of *MINELESS*. In no-choice experiments consumed area of cotyledons increased in correlation with increasing number of days of feeding, and wild-type plants were consumed more than *MINELESS* plants.

The ablation of myrosin cells and consequent lack of myrosinase activity did not affect feeding by pollen beetles in both feeding experiments. Wild-type buds were damaged more than *MINELESS* buds, however the difference was not significant. Also in Y-tube olfactometer experiment beetles moved mostly towards odour of oilseed rape flowers rather than fresh air.

# Table of Contents

# List of Figures

# List of Tables

# Chapter 1

# Introduction

## 1.1 Importance of *Brassica* crops

The Brassicaceae or Cruciferae family, also known as crucifers, contains about 375 genera and 3200 species, which includes crops, ornamentals and weeds. Wild members of the tribe Brassiceae are the sources of oils, condiments and other products and can be a donors to provide useful genes to crop spp. Cultivated species of Brassicaceae include oilseed rape, cabbage, cauliflower, broccoli, Brussels sprouts, turnip, kale, swede, several mustards and other leafy vegetables. These species are grown world-wide under diverse agro-climatic conditions. *Brassica* is economically the most important genus of the tribe Brassiceae belonging to the crucifer family. The genus consists of 159 species along with both cultivated and wild species (Ahuja et al., 2010; Rakow, 2004).

The one of sixteen families of glucosinolate-containing higher plants is *Brassica* genus. Glucosinolate-containing plants (e.g. capers, Capparis spinosa; wasabi, Wasabia japonica; Arugula, Eruca sativa; Radish, Raphanus sativus) were used for food and medicative purposes for several centuries, but right now they are examining for their fungicidal, bacteriocidal, nematocidal and allelopathic characteristics (Fahey et al., 2001).

## 1.2 Oilseed rape (*Brassica napus*) as an important oilseed crop

Oil-palm, soybean, rapeseed and sunflower seed, which are the four most important oil crops, are responsible for 75% of the world production (FAO, 2013).

During the past 30 year rapeseed/canola (mainly *B. napus*) has been an important agricultural product and become the world's third main source of both vegetable oil (after soybean and oil palm) and oil meal (after soybean and cotton). In both Europe and North America oilseed rape is a main cultivable crop. In the EU more than 5 million hectares and in Canada over 6 million hectares area are sown with oilseed rape. There is still increasing demand for oilseed production worldwide (Williams, 2010; Snowdon et al., 2007).

Oilseed rape (*B. napus*) is known as the oldest cultured plant, and it was grown in the Mediterranean regions already 4000 years ago (Hiiesaar et al., 2003). Nowadays oilseed rape (*Brassica napus* L.; genome AACC, 2n = 38) is cultivated more than other crop species existing in the crucifer family (Brassicaceae) (Figure 1.2.1), is separated into two subspecies which both swedes (*B. napus ssp.* napobrassica) and *B. napus ssp. napus* include (Snowdon et al., 2007).



*Figure 1.2.1: Oilseed rape, B. napus, field of Rothamsted Farm, Harpenden, UK.*

Oil is extracted from the remaining meal of small spherical seeds and is made up about 40 % of the seed. The meal contains approximately 40 % protein by weight. The oil has been utilized for different purposes, such as lamp fuel, lubricant, in the chemical industry, etc. Nowadays it is mostly used in cooking or production of food (Lamb, 1989).

The crop has a high yield potential under efficient agronomic cultivation and is used as an important break crop in cereal rotations. Also it has been used as an organic fertilizer, but now mostly is added to animal feed. Various field of use intensifies the value of oilseed rape oil. In addition, its use as a remarkably nutritious food oil, oilseed oil also supplies a raw material for an impressive array of products, along with rapeseed methyl ester (biodiesel) to industrial lubricants, tensides for detergent and soap production and biodegradable plastics (Lamb, 1989; Snowdon et al., 2007; Williams, 2010).

Transforming the chemistry of the seed lead to the increased use of crop, and the consequent increase in production occurred. For human consumption of the oil, erucic acid had a possible

health hazard, for this reason, first the fatty acid composition of the oil was transformed to decrease the level of erucic acid,, and then the levels of glucosinolates present in the meal were reduced (Stefansson et al., 1961). To denote the goal of reduced levels of erucic acid and glucosinolates, which were also repellent or toxic for some farm animals, in the seed to zero, the transformed crop is commonly referred to in Europe as 00 rape, and in Canada this crop is known as Canola®. These improvements in the fatty acid composition of crop have increased consumption of rapeseed oil in human nutrition. Canada's largest biodiesel plant will use canola as a feedstock (Lamb, 1989; Trautwein, 1997; Singh, 2013). Additionally, the consumption of this food oil in Canada is very high, and has been approved for Generally Recognized as Safe (GRAS) status by the Food and Drug Administration (FDA) of the United States Department of Health and Human Services (Dupont et al., 1989). Fatty acid composition of rapeseed oil distinguishes it from the other fats and oils; it is extremely low in saturated fatty acids and is an important source of the monounsaturated oleic acid which is with about 60 % the most abundant fatty acid (Trautwein, 1997).

## 1.3 Glucosinolates

Due to the toxic effects on the vertebrates that feed on the crop plants containing them, glucosinolates have been among the main research subjects starting 1900s. In 1953, researchers ended up with this conclusion that considering role of glucosinolates in host plant selection by crucifier feeding insects is inevitable. It was after 1970s that more thorough studies have been done with regard to this area (Leather, 2014).

Glucosinolates are known to be one of the important sulfur-containing plant secondary metabolites (Figure 1.3.1), mainly because of their existence in the order Capparales. Glucosinolates has been studied in significant crops of brassicaceous plants, cabbage, broccoli, and oilseed rape (canola), or their condiments, such as mustard, horseradish, wasabi, etc. Hydrolysis products of Brassicaceae vegetables such as isothiocyanates give these foods particular taste and flavors. For instance, indole glucosinolates and especially the ones which contain alkenyl R groups cause bitter taste (Giamoustaris and Mithen, 1995; Halkier and Gershenzon 2006; Hopkins et al., 2009; Björkman et al., 2011) .

$$R - C \begin{array}{c} \nearrow N - O - SO_3^- \\ \searrow S - \beta - D - Glucose \end{array}$$
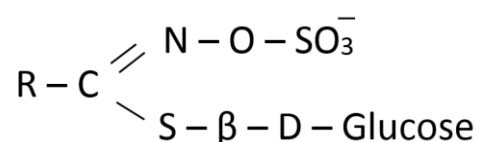
*Figure 1.3.1: The glucosinolate molecule. R denotes the side chain (Giamoustaris and Mithen, 1995).*

The Brassicaceae family is capable of producing special secondary metabolites, the glucosinolates (anionic thioglucosides). The glucosinolates consist of large a class of non-volatile and sulphur-containing secondary plant metabolites, present in all economically important Brassica crops. It is believed that out of consensus structure number of 140 for glucosinolates, 30 of them are present in *Brassica* species (Ahuja et al., 2010).

Over the last 30 years, glucosinolates turn out to be important in agricultural industry, thanks to the increasing significance of cultivars of *B. napus*, *B. rapa*, and *B. juncea* as oil crops in clement and subtropical areas (Fahey et al., 2001).

Oilseed rape meal for livestock holds high protein content which consists of glucosinolates, such as progoitrin or epiprogoitrin, gluconapin, and glucobrassicanapin. Intact glucosinolates have a significant role in feeding and oviposition stimulation (Halkier and Gershenzon, 2006).

Glucosinolate comprises about 1 % of dry weight in several tissues of the *Brassica* vegetables however, their content is noticeably unstable; in some plants it can reach up to 10 % with a possible half of the sulfur content within seeds (Fahey et al., 2001). The glucosinolate content of a plant differs among plant organs, ontogenetic state of plant and agronomic factors related to its growth. Considering the significant role in plant defence, reproductive organs, counting seeds, siliques, flowers and developing inflorescences, followed by young leaves, the root system and fully expanded leaves contain the highest levels of glucosinolates. In seedlings, the cotyledons and in reproductive tissues, flowers and seeds, maintain the highest glucosinolate content. Among vegetative plants, roots contain higher glucosinolate levels than leaves, and among leaves the highest glucosinolate levels belong to the youngest ones. Sulfur-rich S-cells of the flower stalk, which locates near myrosin cells between the phloem and endodermis, maintain remarkably high levels of glucosinolates (Hopkins et al., 2009; Grubb and Abel, 2006; Fenwick and Heaney, 1983).

### 1.3.1 Glucosinolates upon insect attack

Glucosinolates play a role on interaction between Brassicaceae and their related insect herbivores, and avoid pests and diseases from damaged *Brassica* plants. This is done mainly

by the toxic or deterrent effects of glucosinolates as an essential defence mechanism, which made them be known as a class of natural pesticides (Ahuja et al., 2010).

The damage done by pathogens and insects, or mechanical wounding can change concentrations of individual glucosinolates, which may either remarkably increase or decrease glucosinolate levels. Glucosinolates in *B. napus* were induced more when mechanical wounding had been applied for two consecutive days rather than one day (Bodnaryk, 1992). Glucosinolate induction after root and shoot herbivory can be local induction, detected at the site of damage, or systemic induction, detected all over the plant. As an indicator of defence function against insect herbivory, the levels of at least one glucosinolate class increases. Glucosinolate induction can occur within a day, or take place for a long time. As a result of eight weeks feeding, big differences can be detected between control and insect induced plants, which this also can affect herbivores colonizing the same plants later (Hopkins et al., 2009). Glucosinolates play a feeding stimulant role for crucifer-specialists, however they generally exhibit detrimental role for generalist insect herbivores (Bartlet et al., 1999; Hopkins et al., 2009).

*Brassica* plants emit mixtures of volatile organic compounds (VOCs), including alcohols, ketones, aldehydes, esters, terpenoids, sulfides, carboxylic acids, nitriles and isothiocyanates, after being infested by herbivores (van Dam et al., 2012). Glucosinolates and their hydrolysis products generally display unfavorable effects on diverse herbivores, along with mammals, birds, insects, molluscs, aquatic invertebrates and nematodes. These products may exhibit toxicity, may inhibit insect growth, or may prevent feeding by herbivores (Textor and Gershenzon 2009). The studies related to analysis of hydrolysis products show that, these adverse effects are not the role of parent glucosinolates, instead their breakdown products, especially isothiocyanates (Burow et al., 2006).

All glucosinolates do not exhibit equal stimulatory effects, high levels of them can reduce the performance of herbivores that become specialized on brassicaceous species. Insects alter their feeding behaviour according to these variations. Despite having adverse effects of high glucosinolate levels even on specialist insects, adapted insects may explore plant parts with high glucosinolate concentrations in their favour. This strategy gives insects better protection against natural enemy attack, access to plants parts to feed themselves, and ways of escaping from competition (Gols et al., 2008a; Hopkins et al., 2009) . The role of glucosinolates in host plant recognition for feeding and oviposition by specialist herbivores shows that glucosinolates

are not always efficient on anti-herbivore defences. For generalist herbivores glucosinolates are exhibiting defensive features, however act as attractants, especially as an important feeding cues for specialist herbivores, such as *Pieris* spp. caterpillars and *Plutella* spp. (seed weevils, flea beetles) (Textor and Gershenzon, 2009; Fahey et al., 2001).

Root and shoot herbivory can bring about interactions between aboveground and belowground induced defences (Dam et al., 2004). Systemic changes in shoot defence levels may occur as a result of belowground herbivory, which may affect the performance of aboveground herbivores either positively or negatively (Dam et al., 2006).

Increasing levels of glucosinolates correlates with the increasing levels of damage done by the specialist pests. However, the amount of damage caused by the generalist pests decreases with the increasing levels of glucosinolate in the leaves of the oilseed rape plants (Leather, 2014). For instance, increases in the concentrations of glucosinolates lead to the increasing damage by specialist adult flea beetles (*Psylliodes chrysocephala*) and a greater incidence of *Pieris rapae* larvae. Glucosinolates may play a role as feeding and egg laying stimulants for both *P. chrysocephala* and *P. rapae*. Moreover, glucosinolates with difference in their side chain structure may result in different interactions. For example, volatile isothiocyanates derived from short chain aliphatic glucosinolates may have a siginificant role in attracting *P. rapae* adults to the plants, or glucosinolates from leaf surface waxes, such as indolyl glucosinolates may exert a stimulating effect on egg laying, or some other glucosinolates and their breakdown products may stimulate feeding behaviour of larvae (Giamoustaris and Mithen, 1995). Maximum herbivory by the specialist flea beetle *Phyllotreta cruciferae* (Goeze), and diamondback moth, *Plutella xylostella in Brassica rapa* (syn.campestris) occurred with intermediate glucosinolate levels. Counteradaptation was not formed because very high levels of glucosinolates were adverse (Siemens et al., 1996; Bruce, 2014). Study by Ulmer and Dosdall (2006) shows that when feeding, oviposition preference, larval development, and oviposition behavior of the *Brassica* specialist, cabbage seedpod weevil, *Ceutorhynchus obstrictus*, were investigated in eight Brassicaceae species with different glucosinolate profiles, larval growth or development of insect was not affected by total glucosinolate levels. However, there was a correlation between high levels of specific glucosinolates, along with p-hydroxybenzyl and 3-butenyl glucosinolate and prolonged developmental time or decreased weight of insect. When Gols et al. (2008a) compared the development of several specialist and generalist herbivores and their endoparasitoids reared on a wild and cultivated population of cabbage, *B. oleracea*, and a recently established feral *Brassica* spp., wild type population

slowed down the development of herbivores and parasitoids feeding on them regardless of insects species or their dietary specialization. Significantly higher glucosinolate levels were detected in leaves of undamaged cabbage plants, *B. oleracea*, from the wild population than from the domesticated populations. Also herbivory increased total glucosinolate levels significantly in wild plants, but not in domesticated or feral plants.

### 1.3.2 *Delia floralis* and *D. radicum*

*D. floralis* (Fallén), the turnip root fly and *D. radicum* (L), the cabbage root fly, are economically important pests on *Brassica* vegetables, root crops and canola in the north temperate region. On their larval stage *D. floralis* and *D. radicum* feed on the plant roots and cause damage on *Brassica* crops. For this reason eggs and neonates of these herbivores are the part of the life cycle should be controlled most (Klingen et al., 2002).

After exploring the plant by walking on the leaf surface and stem, female flies of cabbage root fly move down onto the soil, and they oviposit in the soil close to the plant stem. Glucosinolates and another group of non-volatile compounds, CIFs (Cabbage Identification Factors), highly stimulate oviposition by cabbage root fly. Hatched larvae feed on the roots and cause damage (Roessingh et al., 1997; de Jong and Städler, 1999).

Insect infestation highly increases plant death rate, and causes decreased marketability even if plant may recover itself afterwards. Plant performance, commercial yields, and defensive chemistries are affected by root herbivores on *Brassica* crops (oil seed rape, kale, swede, canola). Cabbage root fly and turnip root fly damage in *Brassica* spp. roots reducing yield, along with flowering, seed production, leaf, stem and root biomass (Blossey and Hunt-Joshi, 2003). To locate and recognize a proper host, breakdown products of glucosinolates, such as isothiocyanates, and some other volatile compounds play an essential role for cabbage root fly (Ahuja et al., 2010).

### 1.3.3.1 *Effect of Delia herbivory on glucosinolate levels.* Glucosinolates are generally present in the plant at easily measurable levels, and infestation by herbivores stimulates specific glucosinolates by several-fold (Table 1.3.1) (Textor and Gershenzon, 2009). Birch et al. (1996) showed that *D. floralis* infestation on *Brassica* systematically induce special leaf glucosinolates which plays a role as oviposition stimuli. Volatile hydrolysis products of glucosinolates affect selection of oviposition site by *D. radicum*, and purified glucosinolates will stimulate oviposition by *D. radicum* (Hopkins et al., 1997).

*Table 1.3.1. Effects of Delia herbivory on glucosinolate level (Adapted from Textor and Gershenzon, 2009).*

| **Plant species** | **Herbivore species** | **Plant species** | **Herbivore species** |
|---|---|---|---|
| *Brassica napus* L. | *Delia floralis* (Fallen) Diptera, specialist | 1.2-1.35x | Aliphatic (0.75–0.98x) <br> Aromatic (1.5–2.5x) <br> Indolic (2–4x) |
| | | 1.2-1.3x | Indolic (1.6-2.2x) |
| | | 2-3x | Indolic (1.2-5.0x) |
| | | No change | Aliphatic (0.5x) <br> Indolic (2-3x) |
| *Brassica nigra* L. | *Delia radicum* (Linnaeus) Diptera, specialist | No change | Aromatic (0.4–0.9x) <br> Indolic (1.5–2.4x) |
| | | 1.5x | Aliphatic (up to 1.5x) |
| *Brassica oleracea* L. | *Delia floralis* (Fallen) Diptera, specialist | | Indolic (2–4x) <br> Aliphatic (1–2x) |
| | *Delia radicum* (Linnaeus) Diptera, | No change | Aromatic (no change) Indolic (1.9–4.7x) |

## 1.4 Glucosinolate-myrosinase defence system

Plants have developed a wide range of defence mechanisms against the biotic stresses. Glucosinolate–myrosinase system is considered well-known example for these kind of defences, which is also known as "the mustard oil bomb''. Phytochemical features of the order Capparales has long been characterized by glucosinolate–myrosinase system (Bones and Rossiter, 1996; Ratzka et al., 2002) . Myrosin cells are localized in parenchymatous tissue of the green parts of different plants of the Brassicaceae, especially epidermal cells of the leaves (Bones and Rossiter, 1996).

Plants belonging to the order Capparales, including cruciferous crops, are glucosinolates accumulating plants. These plants have myrosinase enzyme with thioglucoside glucohydrolase activity. As a result of hydrolysis of the thioglucoside linkage by the enzyme myrosinase, glucose and an unstable aglycone form (Figure 1.4.1). Glucosinolates and myrosinase are brought together upon tissue disruption by herbivores, myrosinase cleaves glucosinolates into toxic hydrolysis compounds, such as isothiocyanates, nitriles, thiocyanates, oxazolidine-2-thiones and epithionitriles. For many species isothiocyanates (ITCs) are the most prevalent hydrolysis product of glucosinolates, for other plants nitriles are considered the main ones. At a neutral pH, hydrolysis of glucosinolates which possess aliphatic side chain commonly gives ITCs, however in some other conditions, such as acidic pH (pH < 5), or the existence of $Fe^{2+}$ ions promote nitrile and epithionitrile formation in vitro, respectively. As mentioned above, these breakdown products can deter generalist herbivores and attact crucifer specialists (Barth et al., 2006; Halkier and Gershenzon, 2006; Bones and Rossiter, 2006).
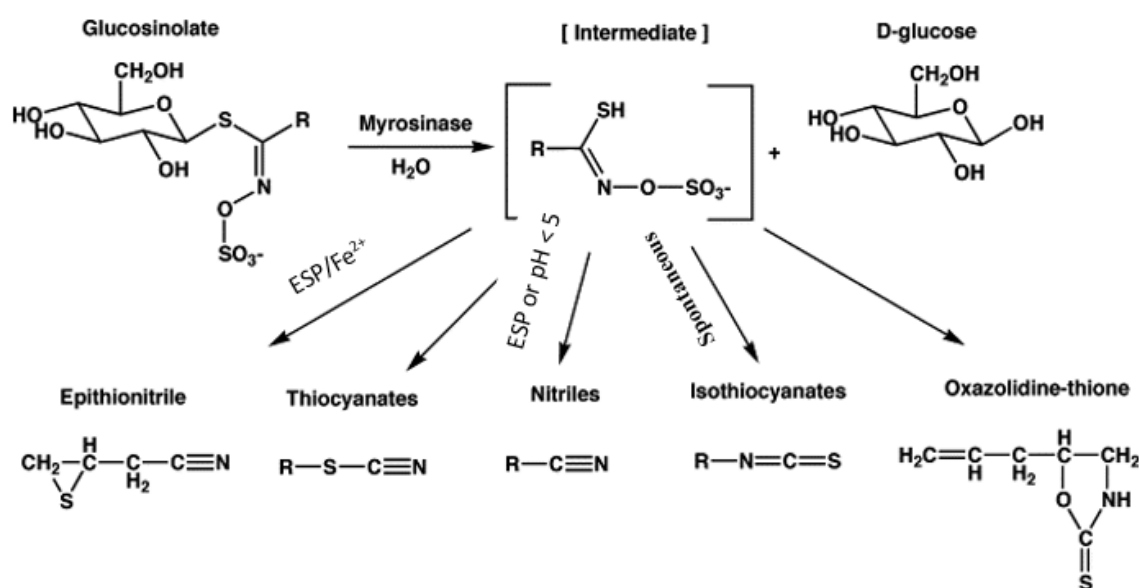


*Figure 1.4.1: The general structure of glucosinolates and their enzymatic degradation products (Adapted from Vaughn and Berhow, 2005; Bones and Rossiter, 2006).*

The nature of the glucosinolates and chemical structure of the side chain mainly affect the chemical product formed by the hydrolysis of glucosinolates. In addition, the reaction conditions, such as the pH, the concentration of ferrous ions, and the existence of epithiospecifier protein (ESP) or epithiospecifier modifier protein (ESM1) also have a role on the result of the myrosinase-glucosinolate reaction (Hopkins et al., 2009; Borgen et al., 2010).

An increase in myrosinase activity might bring about an increase in glucosinolate level, which it will help glucosinolate-containing plants to improve their defensive functions. Additionally, decreasing the hydrolysis rate by inactivation of enzyme myrosinase or breeding for low myrosinase content remarkably decreased toxicity and deterrence of plants towards herbivores (Textor and Gershenzon, 2009).

There are some other proteins which may contribute to glucosinolate hydrolysis like myrosinase. One of these proteins is epithiospecifier protein (ESP) which present in *B. napus* seeds and other members of the Brassicaceae. ESP does not have any catalytic activity in the absence of myrosinase, and brings about the formation of epithionitriles instead of ITCs during the hydrolysis of alkenyl glucosinolates. Nitrile formation occurs by the cleavage of the C–S bond of the glucosinolate aglycone. When the cleaved sulfur reattaches to the terminal olefin, it leads to the formation of epithionitrile (Lambrix et al., 2001).

According to study by Bones and Rossiter (1996) *Sinapis alba* shows 10 times higher myrosinase activity than *Brassica campestris*, and also *B. napus* also possesses slightly higher myrosinase activity than *B. campestris*.

### 1.4.1 Glucosinolate-myrosinase defence system upon insect attack

Upon tissue disruption by insect herbivores, myrosinase hydrolyses glucosinolates to give ITCs, thiocyanates, nitriles, epithionitriles, and oxazolidine-thiones (OZTs) (Bones and Rossiter, 1996, 2006; Grubb and Abel, 2006; Halkier and Gershenzon, 2006; Hopkins et al., 2009). The glucosinolate–myrosinase system acts like a double-edged sword because although they can increase resistance to generalist pests they make plants more susceptible to specialist herbivores (Björkman et al., 2011).

Arabidopsis *tgg1 tgg2* double mutants, which do not have myrosinase enzyme activity in vitro, exhibited reduced breakdown rate of indole glucosinolates in damaged leaf material (Barth et al., 2006).

Myrosinase activity might have more crucial effects on plant defence against specialist insects on Brassicaceae than on generalists, which often do not possess a mechanism to deal with the hydrolysis products of glucosinolates (Li et al., 2000). The defensive function of the glucosinolate-myrosinase system against chewing generalist herbivores depends on glucosinolate breakdown. For instance, according to some studies on free-choice experiments larvae of the generalist *Trichoplusia ni* (cabbage looper, Lepidoptera) feed less on ITCs-

producing than on nitrile-producing Arabidopsis (Wittstock and Burow, 2010). In addition, in no-choice experiments generalist *Spodoptera littoralis* (egyptian cotton leafworm, Lepidoptera), larvae gained weight slower on ITCs- than on simple nitrile-producing lines in the first larval stages. This concludes that ITCs are more efficient against herbivory of generalist Lepidoptera than simple nitriles (Burow et al., 2006).

Although higher glucosinolate levels, faster breakdown rates, and specific chemical structures strongly repelled generalist *T. ni* herbivory, but specialist *P. xylostella* herbivory was not affected by these factors. The study agrees with evolutionary theory stating that specialist herbivores may defeat host plant chemical defences, however generalists will act more susceptible to the same defences (Kliebenstein et al., 2002).

## 1.5  Transgenic *B. napus MINELESS* plants

The word *MINELESS* refers to the genetic ablation of myrosin cells. Myrosin cells were ablated by using the myrosin *Myr1.Bn1* promoter and expressing the cytotoxic RNase barnase in seed myrosin cells. *MINELESS* seeds were produced as a result of controlled cell death (ablation) of myrosin cells, and these seeds possess dramatic reduction of myrosinase-containing toxic mines (Ahuja et al., 2011; Borgen et al., 2010). Transgenic *MINELESS* plants for *B. napus* cv. Westar designated as the wild-type have been developed with the aim of using them as a model to characterize the role of myrosin cells and the glucosinolate-myrosinase defence system against insect herbivores (Borgen et al., 2010).

## 1.6  Cabbage Moth (*Mamestra brassicae*)

The cabbage moth, *Mamestra brassicae*, (Figure 1.6.1) is a generalist herbivore indigenous to the Palearctic, and is often a pest on cabbages and some other crops (Harvey and Gols, 2011). *M. brassicae* (L.) belongs to the family Noctuidae and order Lepidoptera. *M. brassicae* is a polyphagous insect, and has been found on food plants from more than 70 species of 22 families. Among them Brassicaceae and Chenopodiaceae are the most preferred ones by *M. brasicae*. The cabbage moth is broadly spread all over the Europe and Asia and is also native to Norway and is a significant pest on diverse cabbage crops in Southern Norway (Ahuja et al., 2010; Devetak et al., 2010).

*Figure 1.6.1: Larvae of Cabbage Moth (M. brassicae), caterpillars. Picture taken and kindly given by Per Harald Olsens.*

*M. brassicae* has been known as a generalist pest and has been found feeding on a broad range of vegetable plant species. However, among these plant species, *M. brassicae* prefers cruciferous species, and it is mainly considered as a pest of kale and cabbage (Cartea et al., 2010; Rojas et al., 2000). The cabbage moth, *M. brassicae*, generally prefers *Brassica* plants for oviposition and its caterpillars damage cabbage plant (Devetak et al., 2010; Ulland et al., 2008; Johansen, 1997). During a day caterpillars hide themselves under the leaves and in the aboveground plant parts which are close to the soil surface. Larval feeding on aboveground plant parts become more active especially during night and morning hours. Feeding damage can be detected mainly on leaves, flowers of vegetable and sometimes also on fruits of horticultural plants. Damage mainly occurs through larval chewing of leaves at all the stages of the plant growth. Besides mechanical damages through feeding with plant parts, feces of caterpillars on flowers and leaves may also lead to reduced crop quality. As an indirect effects of *M. brassicae* caterpillars on the plant pathogenic fungi and bacteria can be transmitted into damage plant (Devetak et al., 2010; Cartea et al., 2014).

## 1.6.1 Infestation, mortality, behavioural and oviposition experiments with *M. brassicae*

When the mortality of eggs, larvae and pupae and larval dispersal of the cabbage moth, *M. brassicae* (L.) (Lepidoptera: Noctuidae) was investigated in a series of small-scale field experiments and in the laboratory, in white cabbage, *B. oleracea*, the young larvae and hibernating pupae exhibited the highest mortality (Johansen, 1997). Six cabbage (*B. oleracea*)

varieties with different levels of resistance to *M. brassicae* were investigated by Cartea et al. 2014. Larval feeding on leaves from plants at the pre-head stage (from 30-day-old plants after planting) correlated with higher larval mortality, faster larval death, shorter pupation time, lower pupae compared to larval feeding on leaves from plants at the head stage (from 75 day-old plants after planting). For this reason, cabbage plants at their first developmental stages were considered less suitable for larval development of *M. brassicae*.

Natural infestation results in more severe *M. brassicae* damage in outer leaves of *B. oleracea* than in heads because the outer leaves of the plant were preferred by females for oviposition. During artificial infestation, *M. brassicae* larvae were placed into the inner leaves and the larvae consumed more area in heads than in outer leaves. Varieties with more compact heads creates difficulty for larvae to penetrate inside and therefore is considered more resistant (Cartea, 2010).

The orientation and oviposition behavior of *M. brassicae* on cabbage, tomato and chrysanthemum were investigated. The probability of egg laying correlated with the amount of time spent on the plant (cabbage > tomato > chrysanthemum), and matched the oviposition preferences of cabbage moths in choice tests (Rojas et al., 2000). The females with host-plant material, cabbage leaves, laid more eggs than the females without host-plant material. In choice tests, the females oviposited more on cabbage than chrysanthemum, but the mean number of eggs laid on cabbage and tomato was not different (Rojas et al., 2001).

Regarding head foliage consumption and number of larvae per plant, it was concluded that commercial hybrids of cabbage crops to *M. brassicae* were resistant, however local varieties were highly susceptible (Cartea et al., 2010). Less damage were detected by *M. brassicae* caterpillars in the heads of the plants when insecticides had been applied than in the heads of the plants which had been not treated with insecticides. Number of holes in the heads were the highest in the early and mid-early genotypes  (Devetak et al., 2013).

Seljåsen and Meadow (2006) examined the effect of a commercial neem extract on oviposition, and egg and larval development of *M. brassicae* L. on cabbage plants. Neem-treated plants had significantly less number of eggs and egg clusters on them compared to controls. The percentage of one week old hatched larvae was lower in the neem treatment compared to the control. Reduction in damage to neem-treated plants was detected as a result of noticeable reduction in larval development on those plants. The neem treated plants got minimal damage, however the control plants were skeletonized.

## 1.6.2 Effect of *M. brassicae* infestation on glucosinolates

Total glucosinolates content was significantly lower in damaged *B. oleracea* leaves by lepidopterous pests than in undamaged leaves. Sinigrin, glucoiberin, and glucobrassicin were also detected in a lower amounts. These results could be explained by disruption of the leaf tissue by larvae attack and consequent degradation of glucosinolates or by less larval preference of the highest content of sinigrin, glucoiberin, and glucobrassicin (Velasco et al., 2007).

Beekwilder et al. (2008) used *myb28myb29* double mutant, Arabidopsis without any aliphatic glucosinolates, to determine the correlation between aliphatic glucosinolate biosynthesis and herbivory by larvae of the lepidopteran insect *M. brassicae*. The *myb28* mutation blocked the biosynthesis of long-chain aliphatic glucosinolates, and both the *myb28* and the *myb29* single mutants decreased short-chain aliphatic glucosinolates by about 50%. The double mutant did not own any aliphatic glucosinolates, and even severe damage by *Mamestra* larvae on *myb28myb29* double mutant did not bring about biosynthesis of aliphatic glucosinolate.

A study by Velasco et al. (2007) showed that, in damaged *B. oleracea* leaves *M. brassicae* caused 50 % loss of glucosinolates and this decrease affected both aliphatic and indolyl glucosinolates, however *P. xylotella* led to minor reduction, less than 30 % of glucosinolate loss. The lower loss of glucosinolates in *P. xylostella* could be explained by the difference between feeding behaviour of these herbivore. Thus attack by less voracious *P. xylostella* larvae resulted in less damaged area. In the field the specialist *P. xylostella* was more abundant on *P. rapae*-induced plants, and in oviposition experiments *P. xylostella* oviposited more on these plants than on undamaged plants (Poelman et al., 2008). Whereas, the generalist *M. brassicae* showed more abundance on control plants and preferred undamaged plants for oviposition. As a secondary herbivory, the generalist *M. brassicae* affected genes and their expression differently compared to as primary herbivory, thus the expression of *TI* and *TPI* genes, encoding for proteins with trypsin inhibitor activity and a gene involved in glucosinolate biosynthesis (*CYP83B1*) were lower when *M. brassicae* was a secondary herbivore.

Gols et al. (2008b) compared the development of a specialist, *P. rapae* and a generalist, *M. brassicae* insect herbivore on three wild populations and one cultivar of *B. oleracea* under controlled greenhouse conditions. Only development time and pupal mass of *P. rapae* was affected by plant population, however it had a drastic effect on survival of *M. brassicae*. Prolonged development time in *P. rapae* correlated with increased indole glucosinolates, neoglucobrassicin, levels, however decreased survival in *M. brassicae* associated with high

levels of the aliphatic glucosinolates, gluconapin and sinigrin. The explanation for the difference between these species could be the *P. rapae*, the specialist herbivore, is adapted to feed on plants containing glucosinolates and has developed detoxification system against aliphatic glucosinolates.

## 1.7  Pollen beetle, *Meligethes aeneus*, pest status and life cycle

The pollen beetle, *Meligethes aeneus* (Fabricius) (Col.: Nitidulidae), is the major species of pollen beetle found on both winter and spring oilseed rape crops throughout Europe. Early bud stage of crops are most susceptible to damage by the pollen beetle, however as plants develop they become more resistant (Williams 2010; Ekbom, 2010). Increased cultivation of oilseed rape resulted in increased reproductive success of oilseed rape pests, along with pollen beetles (Hokkanen, 2000; Alford et al, 2003).

Pollen beetles are univoltine, and in early spring adults emerge from over-wintering hibernation. Pollen beetles feed on the pollen of spring-flowering plant species from diverse families, whereas they reproduce only on cruciferous plants (family Brassicaceae), including oilseed rape. At this stage, during the summer, to locate new host pollen beetles move from winter-sown to spring-sown crops. Females oviposit in the flower buds (Cook et al., 2002; Blight and Smart, 1999; Williams, 2010). There are two larval instars during pollen beetle development which first instar feed on pollen in the bud, and second instar feed also on open buds and flowers (Osborne, 1965; Williams and Free, 1978; Nilsson, 1988). Both adults and larvae of pollen beetles feed on pollen. Mature larvae move to the soil to pupate. Before migrating to over-wintering sites polen beetles feed on pollen from a broad range of late summer-flowering plant species (Figure 1.7.1). Compared to many herbivorous insects on *Brassica*, adult pollen beetles also feed on non-cruciferous taxa (Cook et al., 2002; Williams, 2010).

The pollen beetle brings about high yield losses in oilseed rape, sometimes it is estimated more than 80 % (Ahuja et al., 2010). Most of the damage to the crop is caused by adult feeding to the young buds of the crop before it comes into flower; the beetles bite into the buds to access the pollen from the developing anthers and damage causes the buds to drop off.  When flowering starts, beetles tend to feed from the pollen easily accessible in the open flowers and the plant is also more able to compensate for any damage at this later growth stage (Williams, 2010; Blight and Smart, 1999, Tatchell, 1983). Because of insecticide resistance, alternative control methods of pollen beetles need to be found.
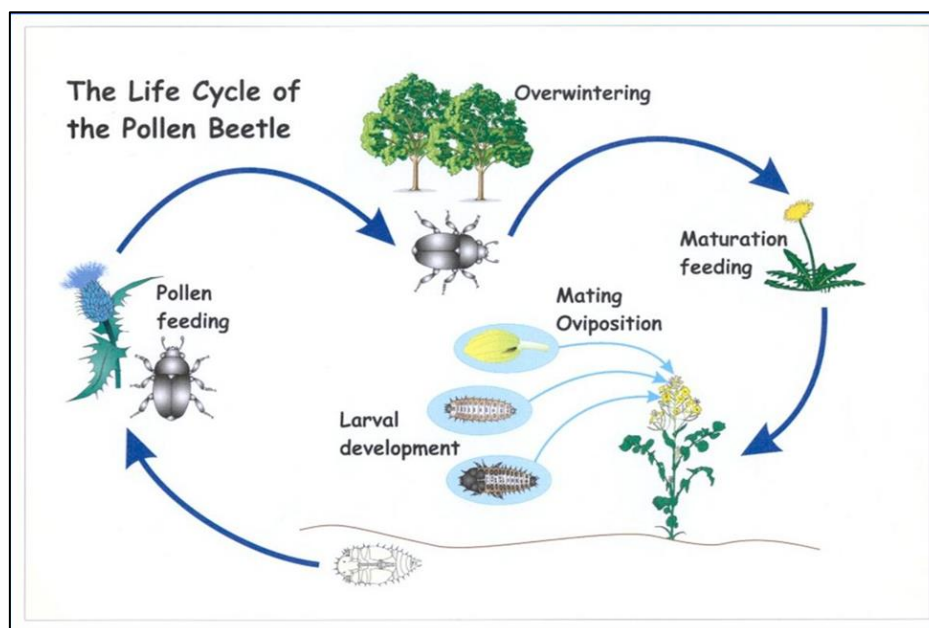
*Figure 1.7.1: Pollen beetle life cycle. Designed by Sam Cook, Rothamsted Research and reproduced courtesy of Rothamsted VCU.*

### 1.7.1 The importance of the glucosinolates to the behavioural ecology of the pollen beetle

After emerging from hibernation sites spring pollen beetles start looking for the host plants (Tölle, 2014). During green bud stage pollen beetles land on oilseed rape crops and feed on pollen in the buds and flowers. With the help of both visual and chemical cues adults can determine their host plants (Williams, 2010). Yellow colour of oilseed rape flowers attracts pollen beetles, therefore floral cues may play an essential role when beetles locate their host plant (Giamoustaris and Mithen, 1996; Blight and Smart, 1999). Since beetles can also locate their host in the early bud stage, when there are not apparent yellow flowers yet, then colour-recognition ability of pollen beetle is not the final mechanism on finding the host (Ruther and Thiemann, 1997).

A study by Cook et al. (2006a) showed that, in polytunnel and field experiments, beetles were attracted more by oilseed rape cultivar "Canyon" compared to "Starlight, which had lower levels of ITCs emission. Also pollen beetles were introduced on both cultivars in different growth stages. This time beetles were more abundant on the cultivar in flower stage than those in bud stage. Thus, polytunnel and field experiments mentioned the importance of relative growth stage in the system. Analysis of volatiles from oilseed rape plants has detected that some volatiles are emitted from only the flowers (Jakobsen et al., 1994). These variation leads to the great diversity in floral scent, which enables insects to identify hosts and non-host plants.

Pollen odour may be used by pollen-seeking insects to locate their hosts. As a result of linear track olfactometer experiments, oilseed rape flowers attracted pollen beetles, which concludes that floral odour can be a basis for these beetles to find their host plants, at least over short distances  (Cook et al., 2002). Also ovipositing female beetles can discriminate between various brassicaceous plant species. Pollen beetles oviposited more into *B. napus* (L.) buds than those of *B. juncea* and *B. campestris* (Ekbom and Borg, 1996). However, pollen beetles did not exhibit any oviposition difference among various *S. alba* populations, even though one population possessed lower total glucosinolate concentration and proportion of sinalbin in its buds than in the other two populations (Hopkins et al., 1998).

Pollen beetles are attracted to some ITCs, particularly 2-phenylethyl isothiocyanate (Blight and Smart, 1999). However, there was not found exact relation between total glucosinolate content of plants and infestation levels by pollen beetles, especially in flowering stage. A series of genetically similar *B. napus* lines with different glucosinolate contents and flower colour were developed to determine the role of glucosinolates and flower colour on herbivory. Before flowering glucosinolates acted as a mild repellent to feeding and there was a significant negative correlation with overall inflorescence glucosinolates. Pollen beetles were significantly more abundant on yellow flower lines than on others, such as on mixed/cream or on white lines, after flowering (Giamoustaris and Mithen, 1996).

More pollen beetles were found on the double-low variety than on the neighboring single-low varieties during an early stage of migration of beetles into a field of oilseed rape in the spring. In addition, pollen beetles were more abundant on open flowers on terminal racemes than on yellow and green flower buds on lateral racemes. Five alkenyl, three indolyl and two aromatic glucosinolates were detected in the floral tissues of oilseed rape where progoitrin (2-OH, 3-butenyl) and glucobrassicanapin (4-pentenyl) were the main compounds. The contents and concentrations of glucosinolates were not different in the terminal and lateral racemes of any variety, and there was only small differences between double- and single-low varieties. No correlation was found between amounts or types of glucosinolates in floral tissues and number and distribution of pollen beetles (Milford et al., 1989).

Detected six volatiles out of the twenty were emitted at higher rates from oilseed rape infested with pollen beetles than those not infested. These compounds were (Z)-3-hexenylacetate, (Z)-3-hexenol, 3-butenyl isothiocyanate and the sesquiterpenes. However infested plants emitted

3-butenyl ITCs, which is derived from 3-butenyl glucosinolate and is the major glucosinolate in buds of oilseed rape, in small but increased amounts (Jönsson and Anderson, 2007).

*Meligethes* spp. are attracted to ITCs, volatile breakdown compounds of glucosinolates. When the release rates were in the range of 5-30 mg/day pollen beetles were attracted equally to four alkanyl, three alkenyl, and to 2-phenylethyl isothiocyanate lures. The addition of a lure consisting of a mixture of allyl, 3-butenyl, 4-pentenyl, and 2-phenylethyl NCS increased the attraction of unbaited yellow traps (Blight and Smart, 1999).

The behavioural response of pollen beetle adults to the odour of their host-plant was studied by using fourarmed airflow olfactometer in the laboratory, and markrelease–recapture technique under semi-natural conditions. Leaves and flowers of oilseed rape were attractive for pollen beetles in the olfactometer. The dispersal of pollen beetles in the presence of yellow water traps, baited with extracts of oilseed rape leaves or flowers, was upwind, and in the absence of oilseed rape odour it was down- and crosswind. Traps baited with rape flower extracts were much more successful on recapturing of pollen beetles than traps with leaf extracts (Evans and Allen-Williams, 1994).

Pollen beetles were less attracted by nitriles than 2-phenylethyl NCS lure. Also it can be inferred that ITCs are more attractive than the 4-pentene- and 5-hexenenitriles. At comparable release rates ITCs are as attractive as 2-phenylethyl NCS. Data from previous field studies shows that specific mixtures of floral volatiles play an important role in maximum attraction by pollen beetles (Smart and Blight, 2000).

It can be inferred form above mentioned studies that role of glucosinolates are unclear, however ITCs in particular are important in host plant attraction. In this thesis the hypothesis explaining *MINELESS* (genetically modified (GM)) plants without myrosinase should produce fewer ITCs and therefore will be less atrractive to pollen beetles than wild type plants has been tested in pollen beetles feeding and in Y-tube olfactometer experiments.

# Chapter

# **Materials and Methods**

## 2.1 Glucosinolate analysis from *Delia radicum* and *D. floralis* challenged material of *B. napus* wild-type and *MINELESS* plants

### 2.1.1 Plant material

Seeds of wild-type and transgenic *MINELESS B. napus* were sown in pots in GMO rooms, Norwegian University of Life Sciences (NMBU), AAS, Norway. The pots were placed in chambers that were kept at 21°C during the day and 16°C at night respectively at a light intensity of 70-80 µmol m$^{-2}$ s$^{-1}$ and 70-80 % humidity. Plants were grown for six weeks.

### 2.1.2 Infestation with *D. floralis* and *D. radicum* larvae and harvest of plants

Five larvae (0-24 h after hatching) of *D. floralis* or *D. radicum* were added to the stem base of each plant (3-4 weeks after germination), when they had 5 to 6 true leaves. Four, ten and fourteen days after infestation with larvae, five-eight biological samples were made from each treatment. Larvae and pupae were retrieved and weighed. After retrieval of the larvae/pupae and scoring of damage, the roots of the plants were quickly washed with water to remove remaining soil, dried with paper tissue and severed from the shoot. The above-ground plant parts (leaves + shoots), and below-ground plant parts (roots) were harvested, photographed, weighed and flash frozen in liquid nitrogen. Each biological sample was comprised of one individual plant. The samples were stored in -80°C freezer for further analysis on glucosinolates. The glucosinolates were extracted and analysed from above-ground tissue of four biological replicas of each of the wild-type and *MINELESS* plants (Table 2.1.1).

*Table 2.1.1. Biological replicas of wild-type and MINELESS plant treatments.*

|  |  | Control (C) |  |
| --- | --- | --- | --- |
|  | Wild-type |  | 4 D |
| *B. napus* |  | *Delia radicum* (DR) | 10 D |
|  | *MINELESS* |  | 14 D |
|  |  | *Delia floralis* (DF) |  |

200 mg of above-ground tissue was weighed, for each sample, transferred to a 2 mL eppendorf tube and immediately flash-frozen in liquid nitrogen for glucosinolate extraction. The unchallenged plants were used as controls. In total, seventy-two samples were used for glucosinolate extraction and further analysis.

### 2.1.3 Extraction of glucosinolates

To extract glucosinolates from *B. napus* wild-type and *MINELESS* samples (aboveground tissues), the extraction method was adapted from Glauser et al. (2012) and Schlaeppi et al. (2008). Before extraction, 2 mL eppendorf tubes with the magnetic beads on the bottom were kept in the liquid nitrogen at -80°C. This was done to avoid thawing of plant material at room temperature, which can cause activation of enzyme myrosinase that would hydrolyse glucosinolates.

Two-hundred mg of frozen above-ground tissue was weighted and added to 2 mL tube. Since *B. napus* does not contain glucosinolate sinigrin, sinigrin was used as an internal standard (IS). Sinigrin containing tube should be shaken few seconds after taking it out of the refrigerator. 1mM 20 µL sinigrin and 980 µL 70% methanol were added immediately. Both sinigrin and methanol should be kept on the ice before and after application. The samples were homogenised with tissuelyser for 1 min at 25 hertz (Hz). After homogenisation, the samples were incubated for 15 min. at 80°C in an incubator to degrade myrosinase. After incubation, the samples were centrifuged at 10,000 rpm for 10 min at 22°C. Each time 1 mL supernatant was transferred to 2 mL tubes and was kept on ice. Then 700 µL supernatant were transferred to 1.5 mL glass vials for further analysis. If the extract is less in amount, then insert can be kept inside the glass vial to make it easier for a needle to take extract.

For the analysis, different concentrations of sinigrin standard were prepared (Figure 2.1.1). The solutions of different concentrations of sinigrin were vortexed for 10 seconds.

| Sinigrin, μL | | MeOH: water (70:30, v/v), μL | | Sinigrin standard, mM/μL |
|---|---|---|---|---|
| 500 | + | 500 | ⟶ | 500 mM, 1000 μL |
| 250 | + | 750 | ⟶ | 250 mM |
| 100 | + | 900 | ⟶ | 100 mM ← |
| 50 | + | 950 | ⟶ | 50 mM |
| 25 | + | 975 | ⟶ | 25 mM |
| 100 | + | 900 | ⟶ | 10 mM ← |
| 50 | + | 950 | ⟶ | 5 mM |
| 30 | + | 970 | ⟶ | 3 mM |
| 15 | + | 985 | ⟶ | 1.5 mM |
| 100 | + | 900 | ⟶ | 1 mM |
| 50 | + | 950 | ⟶ | 0.5 mM |
| 25 | + | 975 | ⟶ | 0.25 mM |
| 10 | + | 990 | ⟶ | 0.1 mM, 1000 μL |

*Figure 2.1.1: Preparation of sinigrin standards of different concentrations.*

## 2.1.4 Glucosinolate analysis

Glucosinolate analysis were performed on a Xevo$^{TM}$ TQ-S (Tandem Quadrupole Spectrometer with Stepwave ion guide) combined with an Acquity UPLC$^{TM}$ from Waters (Milford, MA) (Figure 2.1.2). The Acquity charged surface hybrid (CSH) C18 column (100 mm length x 2.1 mm inner diameter, 1.7μm particle size; Waters) was used.

Glucosinolates were separated using gradient elution using the following mobile phases: mobile phase A = water + 0.05 % formic acid; mobile phase B = acetonitrile (ACN) + 0.05 % formic acid. The following gradient was used for the 11-minutes run: B increased from 2% to

45% in 6 min, then to 100% in 0.5 min; 100% B for 2 min, re-equilibration with 2% B for 2.5 min. A 2μl injection volume was used for the samples.

Instrument was operated in MRM (Multiple Reaction Monitoring) mode. The temperature of the column was maintained at 25°C. The electrospray capillary voltage was set to − 2500 V (2.5 kV) and the cone voltage to −52 V. The source temperature was maintained at 150°C and the desolvation gas tempature at 500°C. The desolvation gas flow was set to 1000 L/Hr. Cone gas flow was 150 L/Hr, nebuliser gas flow was 7.00 Bar. MS inter-scan delay (overhead time) was 0.003 sec. Run time was 11.00 min. Seal wash took 1.0 min; flow rate was 300 μL/min; dwell time was 0.073 sec. Collision energy of 18.0 eV was applied. "ES-" (Electrospray Ionization) ionization mode was used. Argon was used as a collision gas.



*Figure 2.1.2: Xevo^TM TQ-S (Tandem Quadrupole Spectrometer with Stepwave ion guide) combined with an Acquity UPLC^TM from Waters (Milford, MA), Mass Spectrometry Lab of NTNU. 1) Acquity UPLC; 2) Xevo TQ-S.*

## 2.1.5 Data processing

The data was processed using Masslynx^TM v. 4.1. The samples were processed with TargetLynx. The chromatogram window displayed the processed chromatograms associated with the currently selected samples or compounds. Obtained peaks were integrated manually.

Compounds were identified based on their, molecular mass and that of their daughter fragments as reported in Glauser et al. (2012). Sinigrin was used as an IS.

Extracted ion chromatogram of biological replicas WC1 (Wild-type control) and MC1 (*MINELESS* control) run on 17.04.2014 are shown in figure 2.1.3 and 2.1.4, respectively.
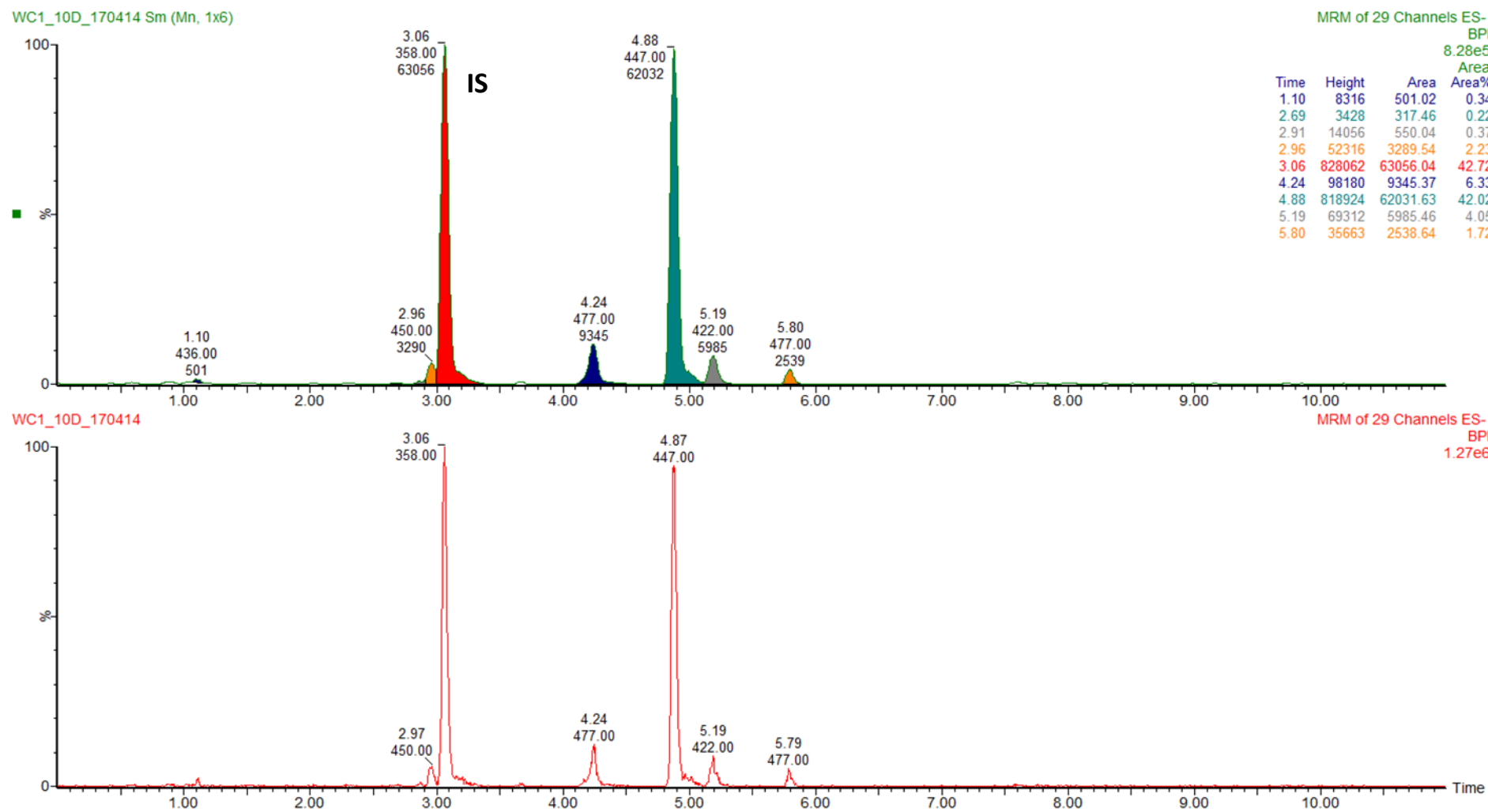
*Figure 2.1.3: Chromatogram window. Extracted ion chromatogram of WC1 (wild-type control-10days) run on 17.04.2014. Chart shows manually integrated peaks of detected compounds in WC1 replica. Peaks represent retention time and mass of each compound. IS, internal standard.*
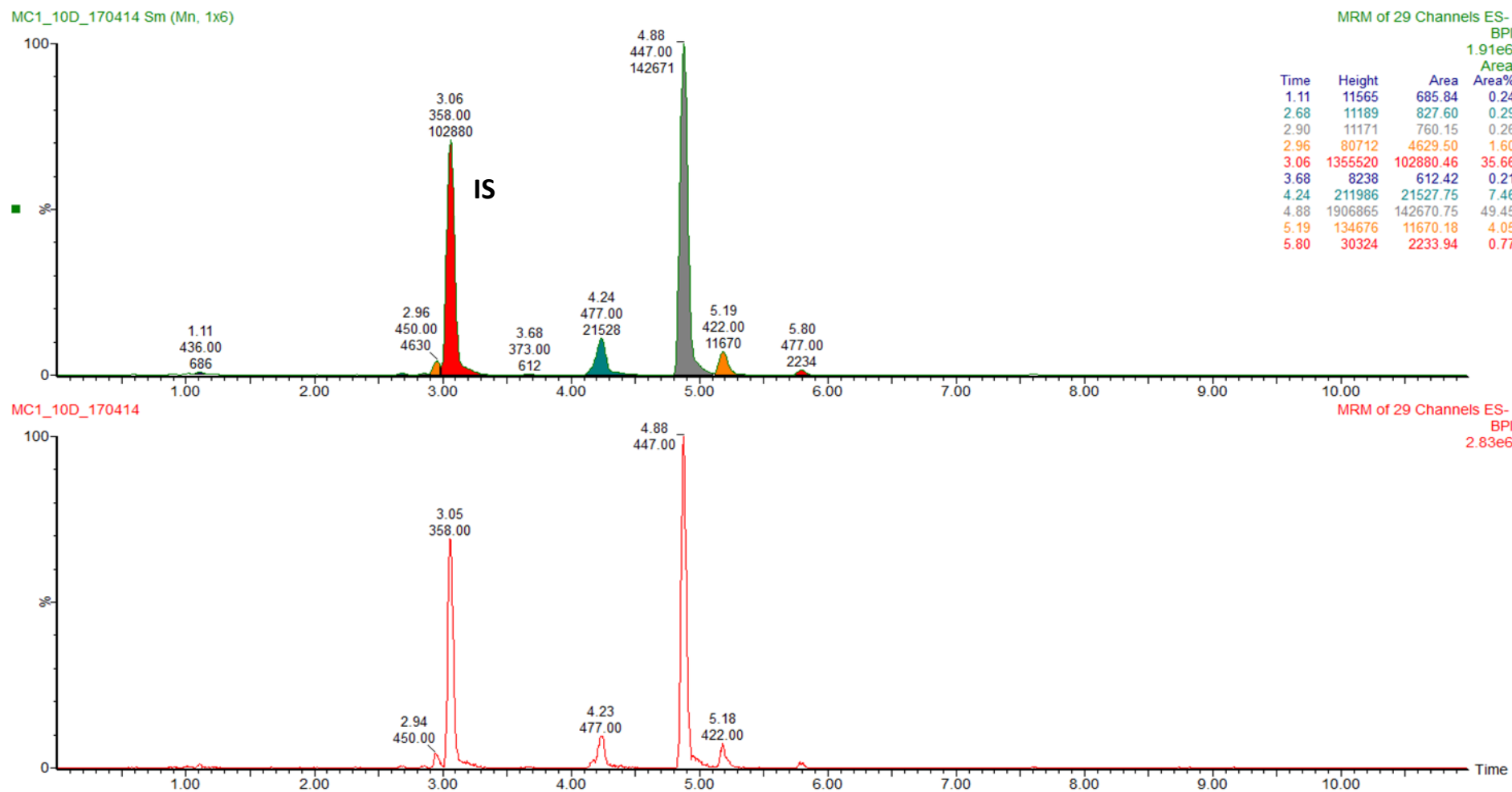
*Figure 2.1.4: Extracted ion chromatogram of replica MC1 (MINELESS control-10days) run on 17.04.2014. Chart shows manually integrated peaks of detected compounds in MC1 replica. Peaks represent retention time and mass of each compound. IS, internal standard.*

## 2.1.6 Quantification of glucosinolates

The glucosinolate quantification was carried out based on added IS. Sinigrin was selected as an appropriate IS because it is naturally absent from *B. napus*. Peak area of each detected compound was normalized to peak area of sinigrin.

$$Glucosinolate\ content = \frac{(Peak\ area\ of\ analyte)}{(Peak\ area\ of\ I.S.)} * 0.02/g\ FW\ of\ sample$$

## 2.2 Consumption of wild-type and *MINELESS* cotyledons of *B. napus* by *Mamestra brassicae* larvae, and growth of *M. brassicae*

### 2.2.1 Plant and insect rearing

The *B. napus* wild-type cv. Wild-type and *MINELESS* seeds were germinated in soil, and plants grown under greenhouse conditions (S3 security class), with a 16 h photoperiod. The day and night temperatures were 21°C and 18°C, respectively at a light intensity of 70-80 µmol m$^{-2}$s$^{-1}$. The eggs of *M. brassicae* were kept at 21°C/16°C; L16:D8.

### 2.2.2 Insect no-choice experiment

Seven-to-nine-day-old wild-type and *MINELESS* seedlings were infested with neonate *M. brassicae* larvae (Cabbage moth; Laboratory of Entomology, Wageningen University). Neonate larvae were weighed to assess their average starting weight and then were distributed over pots (4 seedlings / pot) of each of wild-type and *MINELESS* seedlings. Each seedling was infested with one neonate larvae by placing it on one of the two cotyledons. The larvae were trapped in cages made of opaque plastic sheets. The seedlings were completely enclosed by placing one side of the cage on a pot and covering the other side with a muslin cloth. The experiments were repeated twice. In first experiment, the insect performance was studied for 12 days by taking insect weights at four time points (day- 3, 8, 10, and day 12).

In second experiment, the insect performance was studied for 12 days by taking insect weights at two time points (day 7 and day 12). Larvae were given new seedlings at each time point. The above-ground tissue (cotyledons and hypocotyl) from each seedling was scanned independently and comprised one biological replica. The area of scanned above-ground tissue was calculated with software Compu Eye, Leaf & Symptom Area (Bakr, 2005). To account for

the damage of above-ground tissue during the experiment, the amount of consumed area was calculated by comparison with control above-ground tissue.

### 2.2.3 Insect free choice experiment

Cotyledons from six-seven days old seedlings of wild-type and *MINELESS* seedlings were detached and placed alternatively to each other in 140 mm diameter petri dishes. Four cotyledons of each of the wild-type and *MINELESS* were kept in each petri dish. Wild-type and *MINELESS* cotyledons were kept about 6 mm apart from each other. Neonate *M. brassicae* larvae were raised on artificial diet for three-four days. Seven larvae were kept in the center of each petri dish. Six-eight petri dishes were prepared both with larvae and without larvae (control). The dishes were put in a complete randomized order on trays in growth room and kept at a 16 h photoperiod and day and night temperatures of 21 °C and 18 °C, respectively. After 3 days, the larvae were removed, counted and the cotyledons were collected and scanned. The area of scanned cotyledons was calculated with software Compu Eye, Leaf & Symptom Area (Bakr, 2005). To account for the damage of cotyledons during experiment, the amount of consumed area was calculated by comparison with control (un-challenged) cotyledons. The experiments were repeated two times.

## 2.4 Pollen beetles, *Meligethes aeneus*, feeding and olfactometer experiments

### 2.4.1 Plant and insect rearing

The *B. napus* wild-type cv. wild-type and *MINELESS* seeds were grown individually in 21cm pots containing compost and maintained until use in a completely randomized orientation in a glasshouse (18°C/16°C; with a 16 hour day set for supplementary lighting to come on if required when daylight dipped below 150 umols) (Figure 2.4.1).

Adult pollen beetles were field-collected using a sweep net during June/July, 2014 from spring oilseed rape on Rothamsted Farm, Harpenden, UK. The beetles were a mixture of over-wintered and new generation phenological stage. Collected pollen beetles were transferred to ventilated plastic boxes lined with damp filter paper and were provided with B. napus racemes for food. This was replaced every three days. The insects were kept in plant growth chamber, (Fi-totron), at 10°C, L16:D8 (Figure 2.4.2).

*Figure 2.4.1: Randomized six week old B. napus wild-type and MINELESS plants in glasshouse.*



*Figure 2.4.2: Collecting and culturing pollen beetles at Rothamsted Research. A) Collecting pollen beetles from oilseed rape using a sweep-net; B) Collected pollen beetles are transferred to a tray and captured using an electronic aspirator; C) B. napus racemes collected from the field to feed the beetles; D) Pollen beetles were maintained in controlled conditions in plastic boxes containing with plant material and wet filter paper*

## 2.4.2 Feeding experiment I

Around 150 pollen beetles from the collection were transferred to a new plastic box with wet filter paper and no plant material. Insect were this starved for 48 hours starvation before the experiment to increase their motivation to respond to host plant cues. Plants (32 wild-type and 32 *MINELESS,* were used at the green-yellow bud growth stage (before first flowers were open) and were divided randomly into four treatments (16 plants per treatment):

1. Control undamaged (C.UD)
2. Control damaged (C.D)
3. *MINELESS* undamaged (M.UD)
4. *MINELESS* damaged (M.D)

Damage was applied mechanically to 10 buds on the terminal and first secondary racemes using forceps twenty four hours before the experiment.

The plants were transferred to a controlled environment room maintained at 15°C. Two starved pollen beetles were transferred onto the raceme of each plant and enclosed with a perforated 'bread bag' (10cm x 10cm) with elastic bands (Figure 2.4.2.1). The infested plants were distributed about the controlled environment room into 16 blocks containing one plant of each treatment; however, four of the plants were damaged during transit, resulting in uneven numbers of plants in each treatment.



*Figure 2.4.3: Infesting B. napus wild-type and MINELESS plants with pollen beetles, Meligethes aeneus. A) Infested B. napus plants; B) Raceme part infested with two pollen beetles and enclosed with perforated bag.*

After 24 hours, the bread bag with raceme and pollen beetles inside were excised from each plant. Beetles were retrieved by electronic aspirator and were transferred into labelled vials. The number of beetles retrieved from each plant, whether the beetles were alive or dead was recorded. The beetles were kept in the freezer until the sexes of the beetles could be determined at a later date.

***2.4.2.1 Determining feeding damage to buds.*** The number of beetles on the racemes exposed to the beetles were counted and carefully examined under a binocular microscope. Feeding damage, indicated by rough holes in the buds was recorded. In some cases buds that had been fed upon had opened into flowers but feeding damage was still evident on the sepals and petals.

**2.4.2.1.2 Statistical analysis.** Logistic regression was used to analyze the proportion of buds that had evidence of feeding damage. Terms were included in the model to estimate the effect of replicates, plant treatment (wild-type or *MINELESS*) and plant damage.

***2.4.2.2 Sex determination.*** The beetles were placed dorsally on a microscope slide. A glass cover slip was placed over the ventral side of the insect. By exerting slight pressure onto the cover slip over the abdomen area of the beetle, the triangular-shaped female ovipositor, stylus, and linear-shaped male tegmen and aedeagus, became visible (Cook et al., 2006b).

## 2.4.3 Feeding experiment II

In this experiment, mechanical damage was not applied to buds, and two different treatments were tested, with 32 plants in each treatment:
1. Wild-type (undamaged)
2. *MINELESS* (undamaged)

***2.4.3.1 Infesting plants with pollen beetles.*** The terminal raceme was excised from each plant and were placed into water-saturated Oasis™ floral foam in groups of eight, with three wild-type racemes and 3 *MINELESS* racemes in alternate positions such that there were two racemes of each treatment on each side of the Oasis block. The plants were enclosed by cages comprising Steralin pots with the bases cut off (Figure 2.4.4 A). One pollen beetle (starved for 48h) was placed on each raceme and a ventilated lid fitted. Eight blocks were randomized in the controlled conditions of the Fi-totron (details as above) (Figure 2.4.4 B). After 48 hours feeding damage to buds was determined as described in Experiment I.
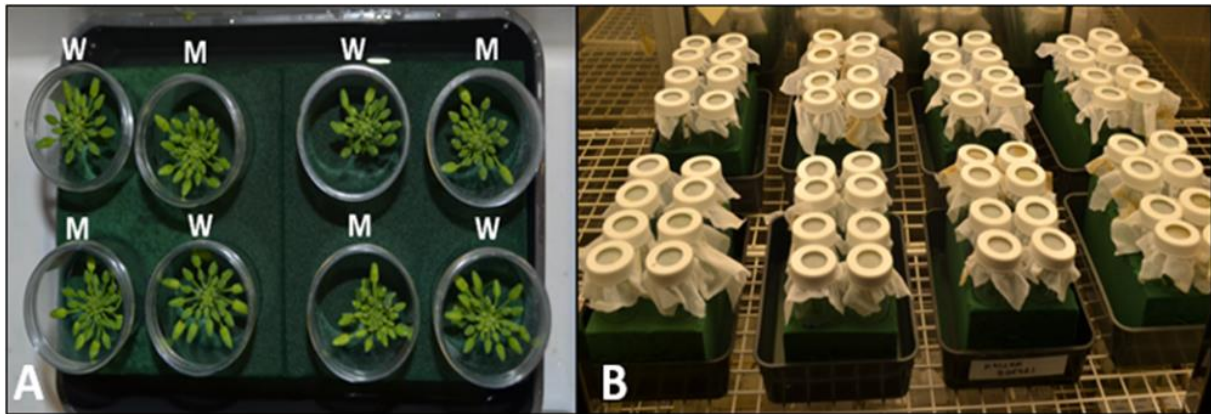
*Figure 2.4.4: Feeding experiment II. A) wild-type (W) and MINELESS (M) racemes in water saturated Oasis™ floral foam in an arranged order. B) Pollen beetle, Meligethes aeneus, infested racemes placed in Fi-totron.*

**2.4.3.1.1 Statistical analysis.** Logistic regression was used to analyse the proportion of buds that had feeding damage. Terms were included in the model to estimate the effect of replicates and plant treatment (wild-type or MINELESS). In the analysis Williams' method (Williams, 1982) was used to control overdispersion (where the variance is found to be greater than expected under the binomial assumptions).

## 2.4.4 Olfactometer experiment

*The Y-tube experimental setup.* An experiment using a Y-tube olfactometer was carried out to examine the orientation responses of pollen beetles to oilseed rape floral odours. The methodology was adapted from Ruther and Thiemann (1997). Experiments were performed in controlled environment room (15°C ± 1°C, mean 80% r.h) which was windowless and painted matt black to reduce visual distractions. The apparatus was lit by an overhead fluorescent light emitting daylight wavelengths and fitted with a plane-polarizing prismatic refractive cover to prodice even illumination. The experimental setup is shown in Figure 2.4.5. The inlets of the Y-tube were connected via silicone tubes with a pump. Airflow was purified through a charcoal-filled gas wash bottle then was split using a polythene adapter into two 500ml glass vessels containing the test materials. The test airflows into the arms of the Y-tube (100 ml/min) were each controlled by flowmeters which were positioned between the vessels and the Y-tube. The Y-tube was fixed on a white plate to maintain it at an angle of, 60°.
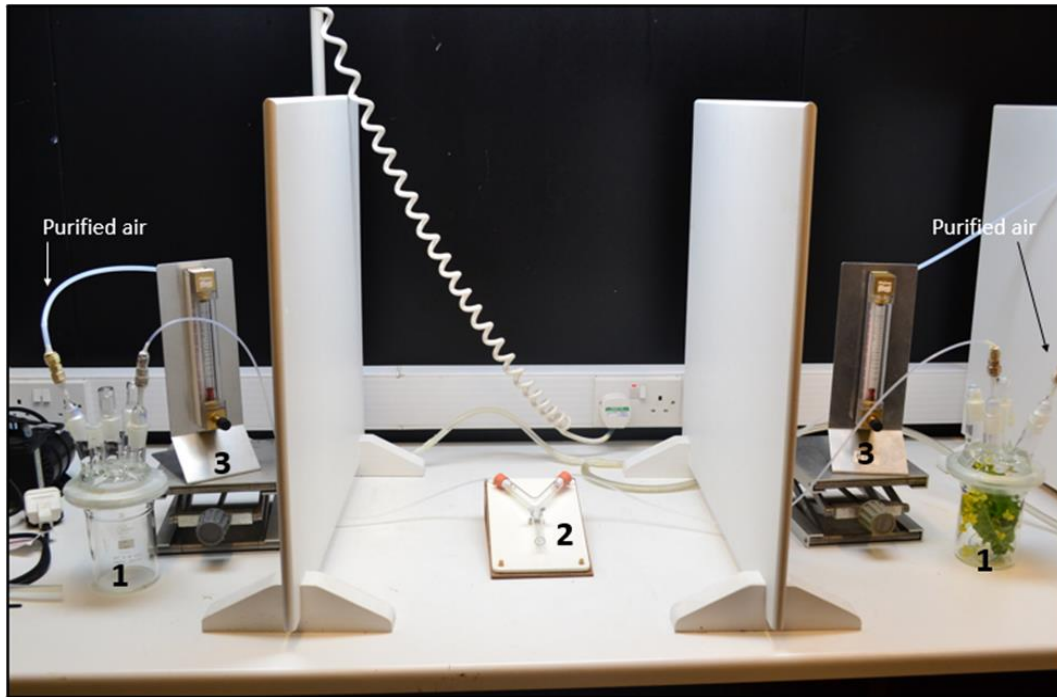
*Figure 2.4.5: Y-tube-olfactometer used in the experiment: (1) glass vessels for plant storage, (2) Y-tube olfactometer, (3) flowmeters.*

To reduce bias  a Y-shaped wire was inserted into the Y-tube, and one beetle (starved for 48h) was released onto the wire at the stem of the olfactometer.  Orientation choice of the beetle was recorded after it had moved  at least 3 cm beyond the junction into one of the arms of the olfactometer (that were  connected with either the test-vessel or the control-vessel). If a choice had not been made after 5 minutes the beetle was classified as a 'non-responder'. The numbers of these individuals were recorded but they were excluded from the final analysis. The wire was changed after each beetle; beetles were used only once.  The orientation of the Y-tube was alternated after every 3 beetles. After every 6 beetles the Y-tube was changed for a clean one and the silicone tubes were connected to the Y-tube the other way around so that the test odour was introduced into the other side of the apparatus.  In this way each odour was introduced into the left and the right sides of the apparatus an equal number of times. Wires were cleaned firstly with acetone, then  rinsed with a 5 % Decon solution in deionized water. The Y-tubes were cleaned for 15 minutes in the Decon solution , and were oven-dried for 30 mins  at 100°C. Beetles were used only once and after responding were collected in individual glass tubes and were frozen so that their sexes could be determined at a later date.

# Chapter 3

# **Results**

## 3.1  Glucosinolate analysis from *Delia radicum* and *D. floralis* challenged material of *B. napus* wild-type and *MINELESS* plants

Tandem Quadrupole Spectrometry (TQ-S MS) was selected for the detection and identification of glucosinolates in *B. napus* wild-type and *MINELESS* extracts. The glucosinolates identified in the extracts from *B. napus* aboveground tissues of wild-type and *MINELESS* are listed in Table 3.1.1. Four different glucosinolates were identified by comparison with IS, sinigrin. Indol-3-ylmethyl- (I3M) (glucobrassicin) was the major glucosinolate in *B. napus*. Other glucosinolates, such as 5-methylsulphinylpentyl- (5MSOP) (glucoalyssin), 4-methoxyindol-3-ylmethyl- (4MOI3M) (4-methoxyglucobrassicin) and 1-methoxyindol-3-ylmethyl- (1MOI3M) (neoglucobrassicin) were also detected in low concentrations (Table 3.1.2).

The trace amounts of other glucosinolates in aboveground tissues of *B. napus* were also detected (3-methylsulphinylpropyl; 4-methylsulphinylbutyl; 2 (R)-2-hydroxy-3-butenyl; 3-butenyl; 2-phenylethyl and 4-hydroxy-indol-3-yl-methyl (4-OH glucobrassicin)).

Table 3.1.1. Glucosinolates identified in B. napus wild-type and MINELESS aboveground tissue. Adapted from Glauser et al., 2012 and Bellostas et al., 2007. Rt, retention time; m/z, mass.

| Desulpho-form | $R_t$ (min) | m/z | Formula | Fragments | Systematic name (abbreviation) | Trivial name |
|---|---|---|---|---|---|---|
| **Aliphatic** | 2.91-3.12 | 450.0564 | $C_{13}H_{24}NO_{10}S_3$ | 386, 192, 96 | 5-methylsulphinylpentyl- (5MSOP) | Glucoalyssin |
| **Indole** | 4.21-4.41 | 477.0633 | $C_{17}H_{21}N_2O_{10}S_2$ | 259, 96 | 4-methoxyindol-3-ylmethyl- (4MOI3M) | Methoxyglucobrassicin |
| | 4.86-5.05 | 447.0530 | $C_{16}H_{19}N_2O_9S_2$ | 259, 205, 96 | Indol-3-ylmethyl- (I3M) | Glucobrassicin |
| | 5.78-5.99 | 477.0632 | $C_{17}H_{21}N_2O_{10}S_2$ | 446, 96 | 1-methoxyindol-3-ylmethyl- (1MOI3M) | Neoglucobrassicin |

*Table 3.1.2. Composition and content of glucosinolates in B. napus aboveground tissues. A) 4 days treatment; B) 10 days treatment; C) 14 days treatment.*

**A**

| *Brassica napus* | Control | | | | Insect Challenged | | | | | | | | | SUM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5MSOP | 4MOI3M | I3M | 1MOI3M | *Delia radicum* | | | | *Delia floralis* | | | | |
| | | | | | 5MSOP | 4MOI3M | I3M | 1MOI3M | 5MSOP | 4MOI3M | I3M | 1MOI3M | |
| Wild-type | 4.4 | 11.8 | 217.4 | 12.8 | 4.5 | 18.3 | 283.5 | 11.0 | 3.3 | 20.8 | 282.1 | 9.1 | 879 |
| *MINELESS* | 3.7 | 23.9 | 310.6 | 4.7 | 4.0 | 20.5 | 321.9 | 5.5 | 4.5 | 25.4 | 382.4 | 5.7 | 1112.8 |

**B**

| *Brassica napus* | Control | | | | Insect Challenged | | | | | | | | | SUM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5MSOP | 4MOI3M | I3M | 1MOI3M | *Delia radicum* | | | | *Delia floralis* | | | | |
| | | | | | 5MSOP | 4MOI3M | I3M | 1MOI3M | 5MSOP | 4MOI3M | I3M | 1MOI3M | |
| Wild-type | 6.1 | 16.3 | 131.9 | 9.7 | 17.1 | 34.7 | 262.6 | 21.2 | 17.7 | 25.8 | 169.8 | 14.4 | 727.3 |
| *MINELESS* | 10.2 | 26.3 | 135.1 | 4.6 | 9.8 | 25.5 | 172.7 | 3.6 | 3.8 | 33.8 | 186.4 | 6.4 | 618.2 |

**C**

| *Brassica napus* | Control | | | | Insect Challenged | | | | | | | | | SUM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5MSOP | 4MOI3M | I3M | 1MOI3M | *Delia radicum* | | | | *Delia floralis* | | | | |
| | | | | | 5MSOP | 4MOI3M | I3M | 1MOI3M | 5MSOP | 4MOI3M | I3M | 1MOI3M | |
| Wild-type | 28.2 | 17.4 | 57.0 | 5.3 | 16.9 | 23.4 | 118.2 | 5.8 | 8.7 | 24.5 | 129.8 | 9.3 | 444.5 |
| *MINELESS* | 17.3 | 15.4 | 68.7 | 1.8 | 16.3 | 20.9 | 119.9 | 3.2 | 12.4 | 26.2 | 125.7 | 3.5 | 431.3 |

*a - concentrations in nmoles per gram.*

### 3.1.1 5-methylsulphinylpentyl- (5MSOP)

5MSOP, glucoalyssin, levels were lower in *B. napus* aboveground tissues when compared with other detected glucosinolates (Figure 3.1.1). Four days treatment showed lower, and 14 days infestation showed higher glucoalyssin levels for both control and insect challenged tissues (Figure 3.1.1 A and 3.1.1 C).

Wild-type *D. radicum* and *MINELESS D. floralis* had the highest 5MSOP levels within four days of infestation. Wild-type tissues showed nearly similar concentrations for glucosinolate 5MSOP. 5MSOP levels were lower in wild-type *D. floralis* challenged tissues, however the control and *D. radicum* challenged tissues had almost the same glucosinolate levels. In *Delia* spp. challenged *MINELESS* tissues higher 5MSOP levels were detected than control tissues (Figure 3.1.1 A).

Within ten days treatment the highest 5MSOP levels were detected in *D. floralis* challenged wild-type tissues, and the lowest concentration was in *D. floralis* challenged *MINELESS* tissues (Figure 3.1.1 B). About three times more 5MSOP was detected in both *Delia* spp. challenged wild-type tissues compared to control. Although *MINELESS* control tissues had higher 5MSOP content than wild-type control tissues, glucosinolate content was lower in Delia spp. challenged *MINELESS* tissues (Figure 3.1.1 B).

Within fourteen days treatment, the highest 5MSOP level was detected in wild-type control aboveground tissues, whereas wild-type *D. floralis* had the lowest 5MSOP content, 3.2 times lower than wild-type control (Figure 3.1.1 C). Both wild-type *Delia* spp. challenged tissues showed decreased 5MSOP levels compared to the control tissues. In control *MINELESS* tissues, 5MSOP concentration was low, and the *Delia* spp. challenged tissues had lower 5MSOP levels than control tissues, but there was no big difference (Figure 3.1.1 C).

**A**

### 5MSOP glucosinolate content, 4 days



**B**

### 5MSOP glucosinolate content, 10 days



**C**

### 5MSOP glucosinolate content, 14 days

*Figure 3.1.1: 5-methylsulphinylpentyl (5MSOP) levels in control (unchallenged) and D. radicum and D. floralis challenged wild-type and MINELESS plants. Values represent mean (±SE) of four biological replicates. (A) 4 days after infestation, (B) 10 days after infestation and (C) 14 days after infestation. WC, Wild-type control; WDR, Wild-type D. radicum challenged; WDF, Wild-type D. floralis challenged; MC, MINELESS control; MDR, MINELESS D. radicum challenged; MDF, MINELESS D. floralis challenged.*

## 3.1.2 4-methoxyindol-3-ylmethyl- (4MOI3M)

The content of glucosinolate 4MOI3M in the aboveground tissues of wild-type control oilseed rape was 11.8 n mol/g f.w. (Figure 3.1.2 A). Four days tissues of wild-type oilseed rape challenged by *D. radicum* and *D. floralis* contained 1.5 and 1.8 times more 4MOI3M, respectively. *MINELESS* control plant tissues possessed about two times more 4MOI3M when compared to wild-type control. Glucosinolate concentrations relatively decreased in four days *D. radicum* challenged *MINELESS* tissues, and increased in *D. floralis* challenged ones compared to control. Overall, both control and four days insect challenged tissues of *MINELESS* aboveground tissues had higher 4MOI3M content than wild-type control and challenged tissues (Figure 3.1.2 A).

After ten days of treatment the 4MOI3M levels were highest in wild-type *D. radicum* challenged aboveground tissues, and the lowest concentration was in wild-type control tissues (Figure 3.1.2 B). Both wild-type *D. radicum* and *D. floralis* challenged tissues showed higher glucosinolate levels than control. *MINELESS* control had 1.6 times higher 4MOI3M levels than wild-type control. *MINELESS D. radicum* challenged tissues had less 4MOI3M concentrations when both were compared with *MINELESS* control tissues and wild-type *D. radicum* challenged tissues. However, *MINELESS D. floralis* challenged tissues showed different results asit had 4MOI3M levels both higher than *MINELESS* control and wild-type *D. floralis* challenged tissues (Figure 3.1.2 B).

After fourteen days treatment the highest 4MOI3M levels were observed in *MINELESS D. floralis* challenged tissues, and the lowest levels were in *MINELESS* control tissues (Figure 3.1.2 C). Both in wild-type and *MINELESS* tissues the infestation by both *Delia* caused higher glucosinolate concentrations compared with control plants.

## 4MOI3M glucosinoate content, 4 days



**A**

## 4MOI3M glucosinolate content, 10 days



**B**

## 4MOI3M glucosinolate content, 14 days



**C**
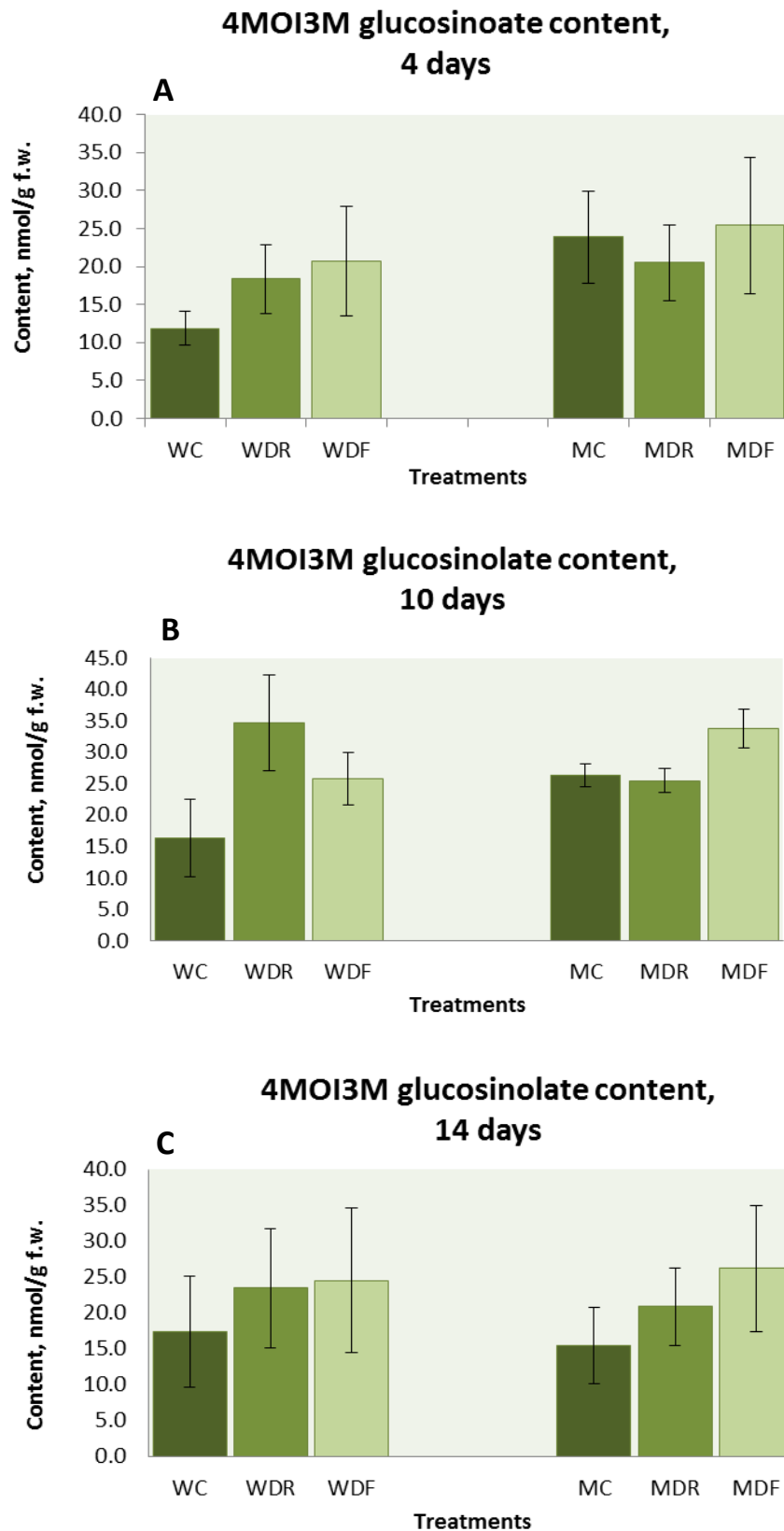
*Figure 3.1.2: Content of 4MOI3M levels in control (unchallenged) and D. radicum and D. floralis challenged wild-type and MINELESS plants. Values represent mean (±SE) of four biological replicates. (A) 4 days after infestation, (B)10 days after infestation and (C) 14 days after infestation. WC, Wild-type control; WDR, Wild-type D. radicum challenged; WDF, Wild-type D. floralis challenged;, MC, MINELESS control; MDR, MINELESS D. radicum challenged; MDF, MINELESS D. floralis challenged.*

39

### 3.1.3 Indol-3-ylmethyl (I3M)

*MINELESS D. floralis* challenged tissues had the highest, while wild-type control tissues had the lowest I3M content within four days of infestation (Figure 3.1.3 B). Both *Delia* spp. challenged wild-type aboveground tissues had higher glucobrassicin levels than control tissues. I3M content in control and challenged *MINELESS* tissues was higher than the wild-type tissues. Again here *MINELESS* control plant tissues showed lower glucosinolate content than *Delia* spp. challenged tissues (Figure 3.1.3 A).

After ten days of infestation, *D. radicum* challenged wild-type tissues showed two-fold increase compared to the control (Figure 3.1.3 B). *D. floralis* challenged wild-type tissues also possessed higher I3M content than control plants, but this is not big difference. Control *MINELESS* plants had almost the same I3M content like wild-type plants. *Delia* spp. challenged tissues showed slight increase compared to the control tissues. Big difference is only observed between wild-type and *MINELESS D. radicum* challenged tissues (Figure 3.1.3 B).

After fourteen days of infestation, there was not big difference on detected I3M content between wild-type and *MINELESS* (Figure 3.1.3 B). *Delia* spp. challenged tissues showed approximately two times higher I3M content than control plant tissues (Figure 3.1.3 C).

**I3M glucosinolate content, 4 days**

**I3M glucosinolate content, 10 days**

**I3M glucosinolate content, 14 days**
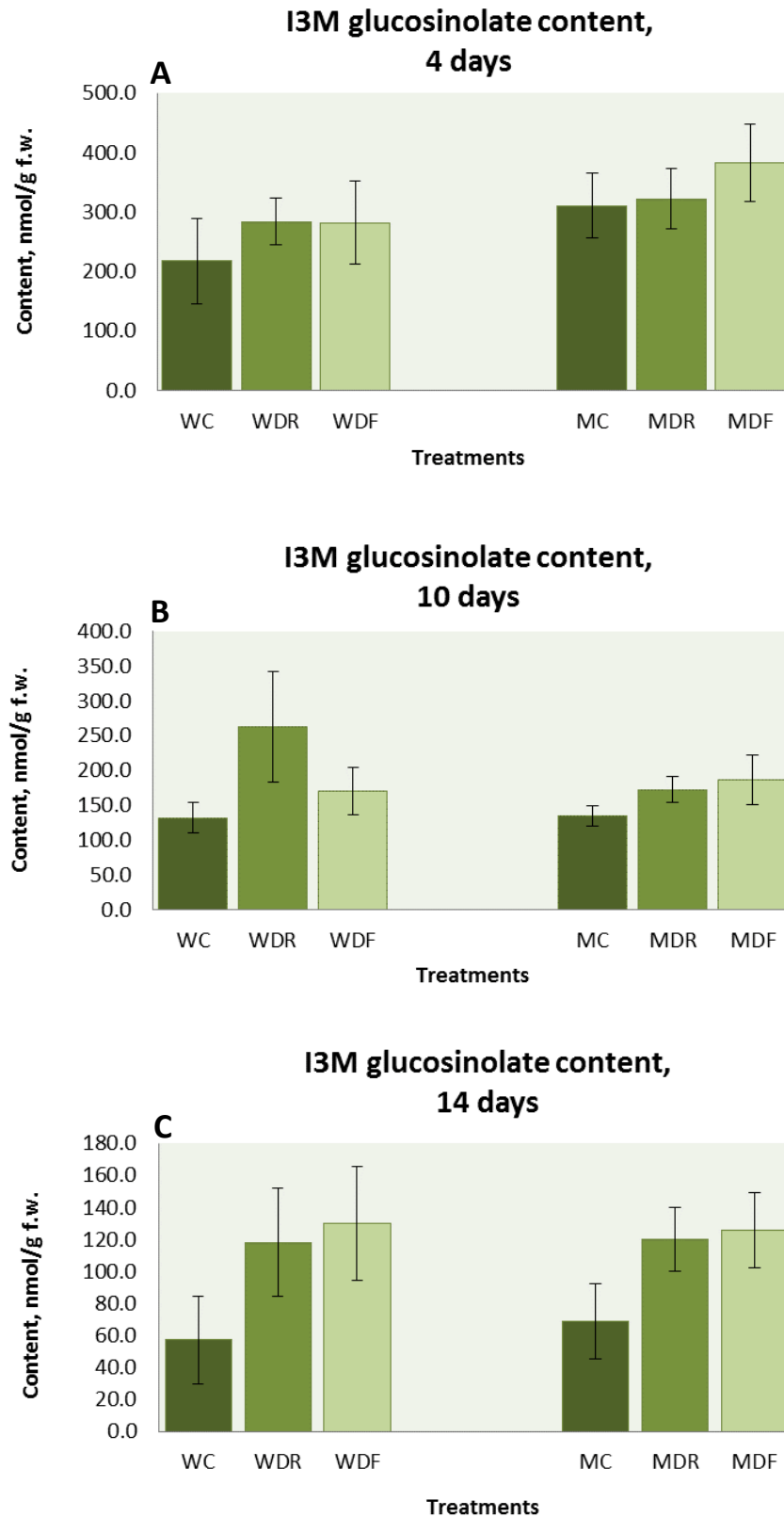
*Figure 3.1.3: Indol-3-ylmethyl- (I3M) levels in control (unchallenged) and D. radicum and D. floralis challenged wild-type and MINELESS plants. Values represent mean (±SE) of four biological replicates. (A) 4 days after infestation, (B) 10 days after infestation and (C) 14 days after infestation. WC, Wild-type control; WDR, Wild-type D. radicum challenged; WDF, Wild-type D. floralis challenged;, MC, MINELESS control; MDR, MINELESS D. radicum challenged; MDF, MINELESS D. floralis challenged.*

### 3.1.4  1-methoxyindol-3-ylmethyl- (1MOI3M)

1-methoxyindol-3-ylmethyl- (1MOI3M), neoglucobrassicin, was detected in lower amount compared to other detected glucosinolates from same tissues (Table 3.1.2).

After four days infestation, the highest 1MOI3M content was detected in wild-type control, while the lowest content was detected in *MINELESS* control aboveground tissues (Figure 3.1.4 A). There was no difference in  content of 1MOI3M between control and *Delia* spp.  challenged tissues. For wild-type plant tissues, 1MOI3M content was slightly decreased in both *Delia* spp. challenged tissues compared to the control tissues. However, there is slight increase in challenged tissues of *MINELESS* plants compared to the control tissues (Figure 3.1.4 A).

After ten days infestation, the highest 1MOI3M content was detected in wild-type *D. radicum* challenged tissues, the lowest was detected in *MINELESS D. radicum* challenged ones (Figure 3.1.4 B). 1MOI3M content in wild-type *D. radicum* challenged tissues showed two-fold increase compared to the control tissues. The content of 1MOI3M in both wild-type *Delia* spp. challenged tissues were higher than control tissues. Control and challenged tissues of *MINELESS* plants were lower in 1MOI3M content than the wild-type plants. The biggest difference was observed between wild-type and *MINELESS D. radicum* challenged tissues, where insect challenged wild-type aboveground tissues showed induction of about six times more 1MOI3M content than *MINELESS* tissues (Figure 3.1.4 B).

Fourteen days of infestation showed difference between the wild-type and *MINELESS* plants (Figure 3.1.4 C). For control plants, three times more 1MOI3M was detected in wild-type than in *MINELESS*. Although *D. radicum* infestation in wild-type plants did not show big difference on 1MOI3M content compared to control plants, but *D. floralis* challenged tissues possessed 1.8 times higher levels of 1MOI3M than control tissues. A slightly higher 1MOI3M was observed in *MINELESS* challenged tissues than in  control tissues (Figure 3.1.4 C).

## 1MOI3M glucosinolate content, 4 days



**A**

## 1MOI3M glucosinolate content, 10 days


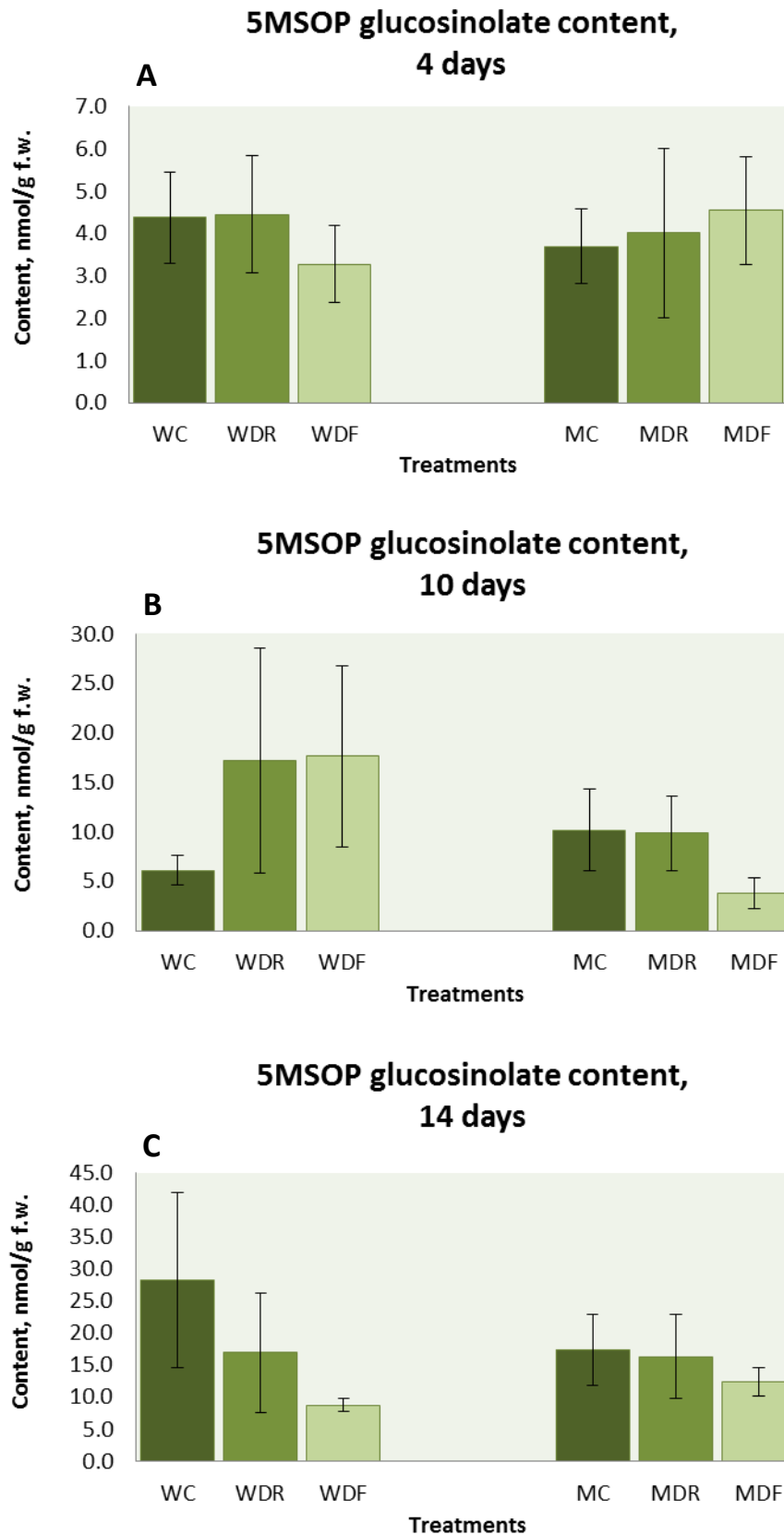
**B**

## 1MOI3M glucosinolate content, 14 days



**C**
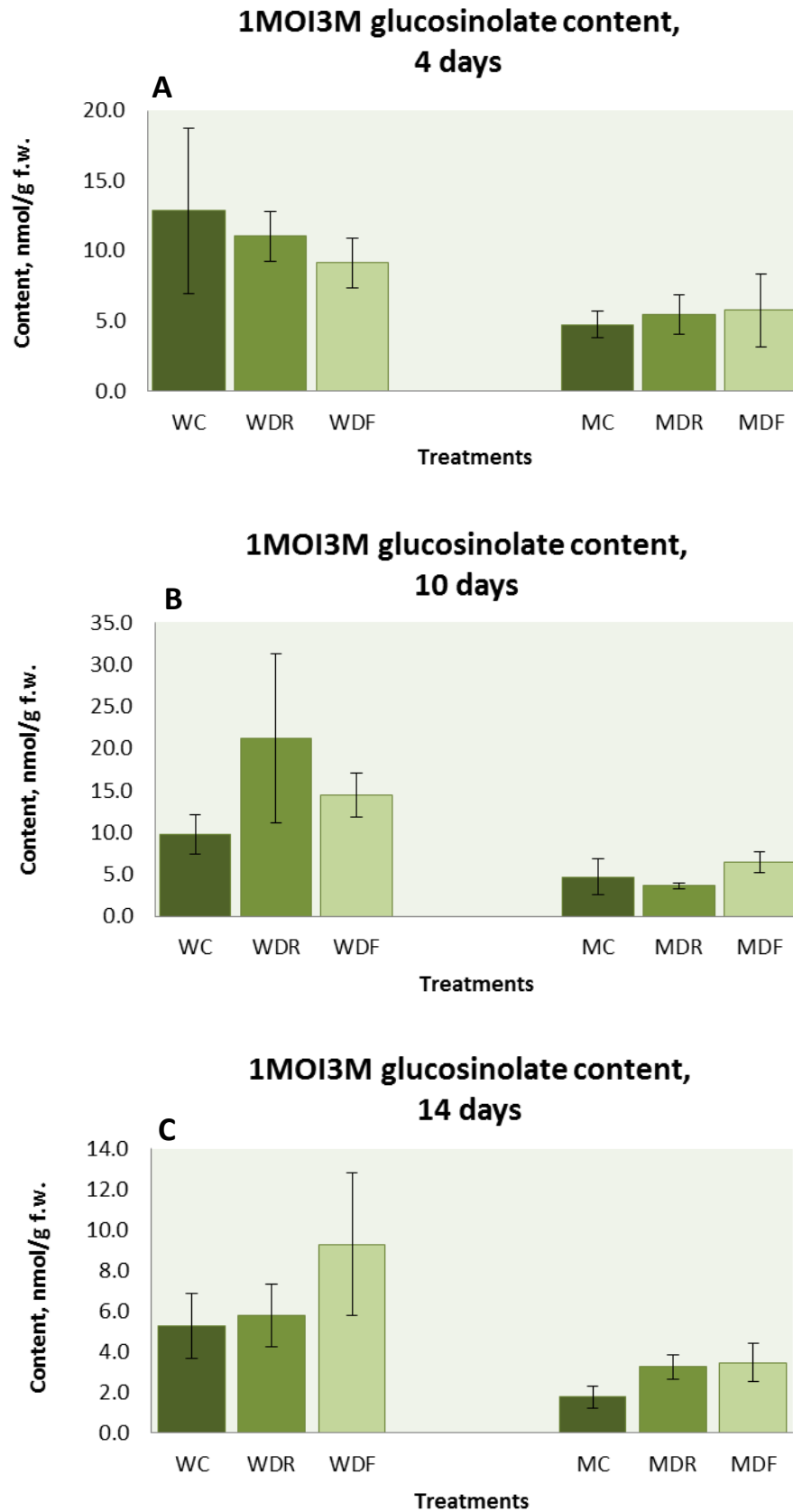
*Figure 3.1.4 1-methoxyindol-3-ylmethyl- (1MOI3M) levels in control (unchallenged) and D. radicum and D. floralis challenged wild-type and MINELESS plants. Values represent mean (±SE) of four biological replicates. (A) 4 days after infestation, (B) 10 days after infestation and (C) 14 days after infestation. WC, Wild-type control; WDR, Wild-type D. radicum challenged; WDF, Wild-type D. floralis challenged;, MC, MINELESS control; MDR, MINELESS D. radicum challenged; MDF, MINELESS D. floralis challenged.*

## 3.2  Consumption of *B. napus* cotyledons by *Mamestra brassicae*

### 3.2.1  Free-choice feeding experiments

In free-choice experiments (Figure 3.2.1) a big difference was observed in consumption of wild-type and *MINELESS* cotyledons by *M. brassicae* larvae. In experiment I, the consumption of wild-type cotyledons by *M. brassicae* larvae was 2.4 times more than those of *MINELESS* cotyledons (Figure 3.2.2 A). Experiment II also showed more consumption of wild-type cotyledons, about 1.5 fold more wild-type cotyledon fed by larvae than those of *MINELESS* cotyledons (Figure 3.2.2 B).  Both experiments showed that *M. brassicae* larvae feed wild-type cotyledons more than *MINELESS* cotyledons.
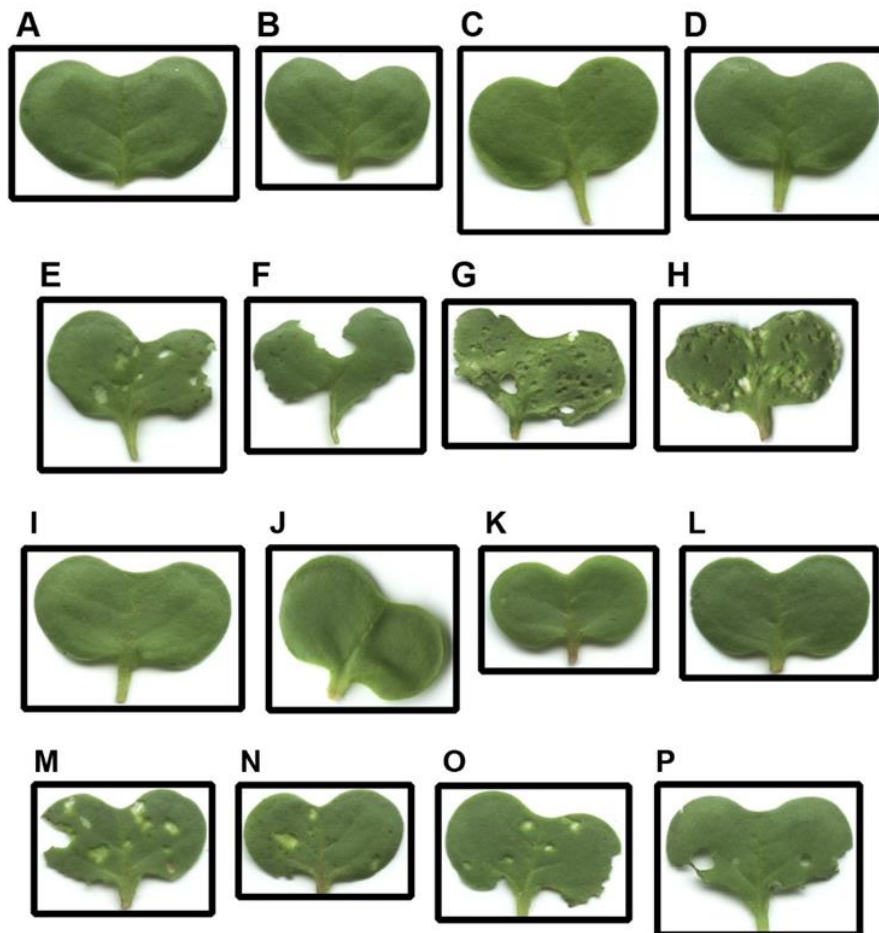


*Figure 3.2.1: M. brassicae damaged cotyledons of wild-type and MINELESS from insect free-choice test experiments. (A-D) Wild-type control cotyledons; (E-H) Wild-type damaged cotyledons; (I-L) MINELESS control cotyledons; (M-P) MINELESS damaged cotyledons.*
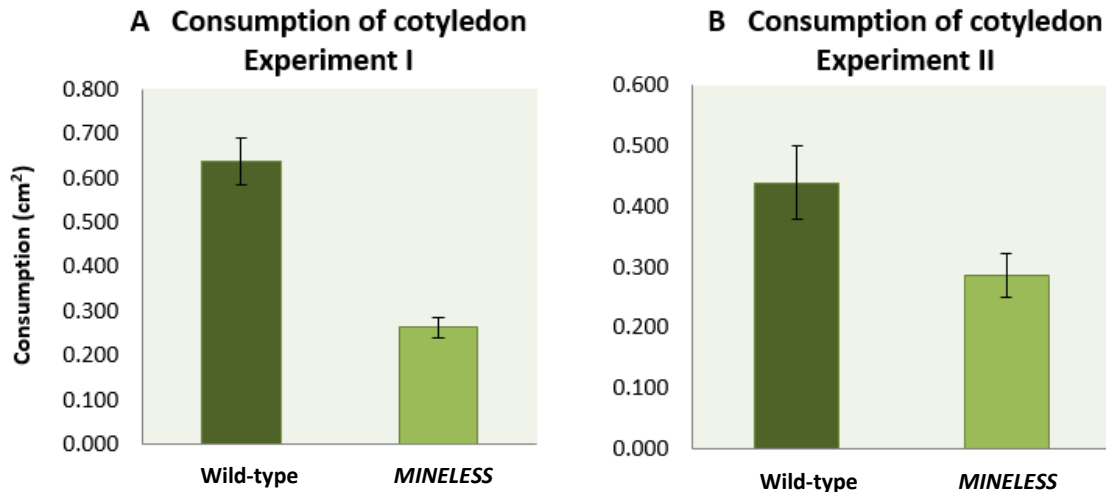
*Figure 3.2.2: Consumption of wild-type and MINELESS cotyledons during free-choice feeding experiments.*

### 3.2.2 No-choice feeding experiments

*3.2.2.1 Consumption of aboveground tissues by M. brassicae larvae.* In no-choice feeding experiments, *M. brassicae* larvae have been feeding on aboveground tissues for different period of time. In experiment I, the consumption of tissue by *M. brassicae* larvae was observed for 3, 8, 10 and 12 days (Figure 3.2.3). For all time periods, larvae consumed more wild-type aboveground tissue than those of *MINELESS* aboveground tissue. Feeding experiment after three days showed less consumption of both wild-type and *MINELESS* tissues by larvae. Tissue consumption by larvae was increased with increase in number of days of feeding. This increase was considerable in wild-type tissues, however was less in *MINELESS*. As a result of maximum feeding after 12 days, tissue consumption was decreased compared to 8 days and 10 days feeding, but still was higher than 3 days feeding. Maximum consumption of wild-type aboveground tissue was as a result of 10 days feeding, for *MINELESS* it was after 12 days larval feeding. Overall *M. brassicae* larvae fed on 1.4-2.6 times more wild-type aboveground tissues than *MINELESS* tissues (Figure 3.2.4).

In experiment II, the consumption of aboveground tissue as a result of after 7 days and 12 days larval feeding was compared between wild-type and *MINELESS* plants. Wild-type aboveground tissues were consumed almost equally by larvae within after 7 days and 12 days. However, 12 days larval feeding caused 1.8 times more tissue consumption compared to 7 days feeding on *MINELESS*. Moreover, there was a big difference between on wild-type and *MINELESS* tissues on 12 days feeding on wild-type and *MINELESS* tissues, 2.5 fold more

tissue consumed by larvae on wild-type plants. Also 7 days feeding experiment after 7 days showed slight difference between wild-type and *MINELESS* tissues.
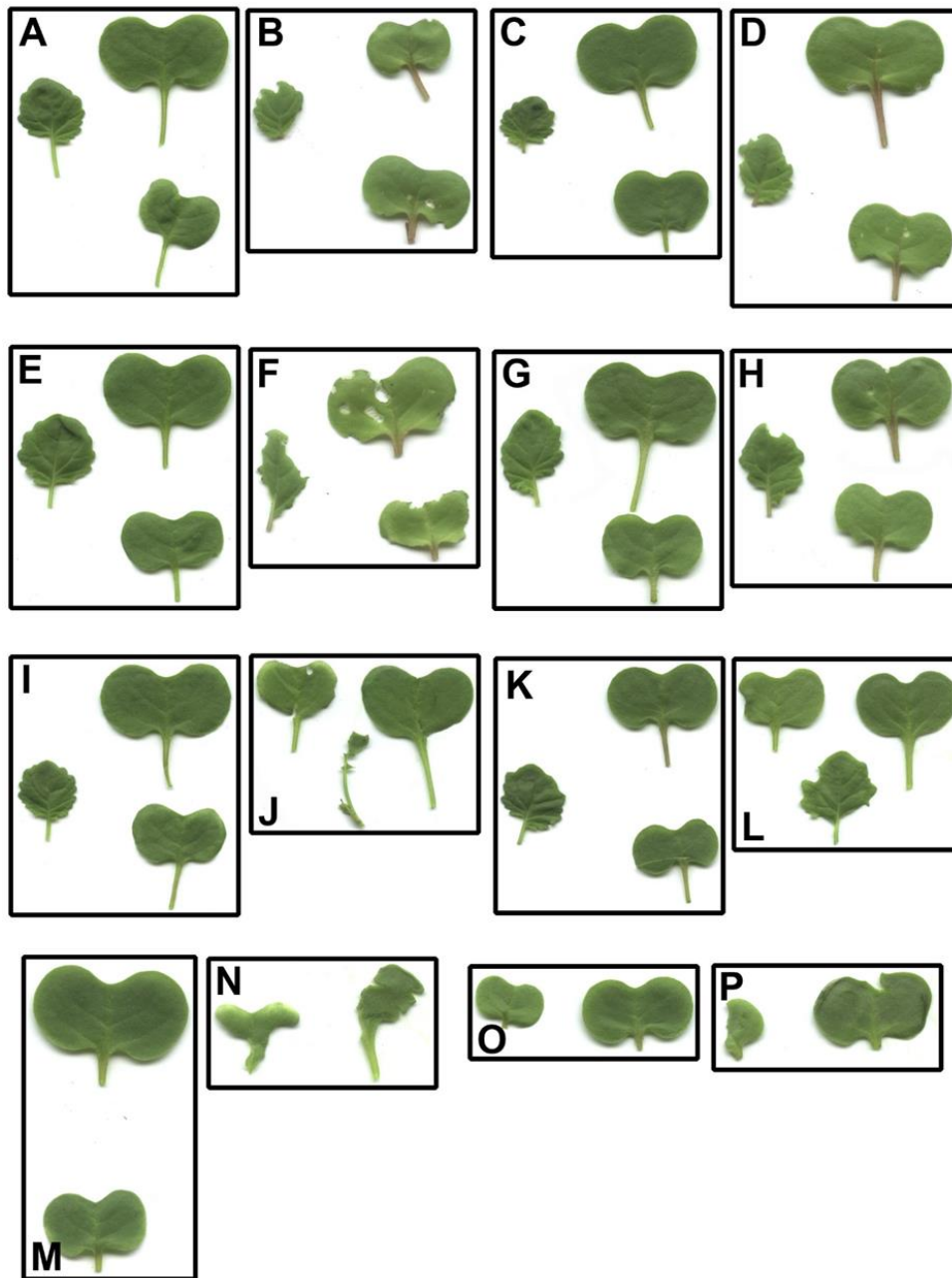


*Figure 3.2.3: M. brassicae damaged cotyledons of wild-type and MINELESS seedlings from no-choice feeding experiments. (A, B) Wild-type control (A) and damaged cotyledons (B) from day 3; (C, D) MINELESS control (C) and damage cotyledons (D) from day 3; (E, F) Wild-type control (E) and damaged cotyledons (F) from day 8; (G, H) MINELESS control (G) and damaged cotyledons (H) from day 8; (I, J) Wild-type control (I) and damaged cotyledons (J) from day 10; (K, L) MINELESS control (K) and damaged cotyledons (L) from day 10; (M, N) Wild-type control (I) and damaged cotyledons (J) from day 12; (O, P) MINELESS control (K) and damaged cotyledons (L) from day 12.*
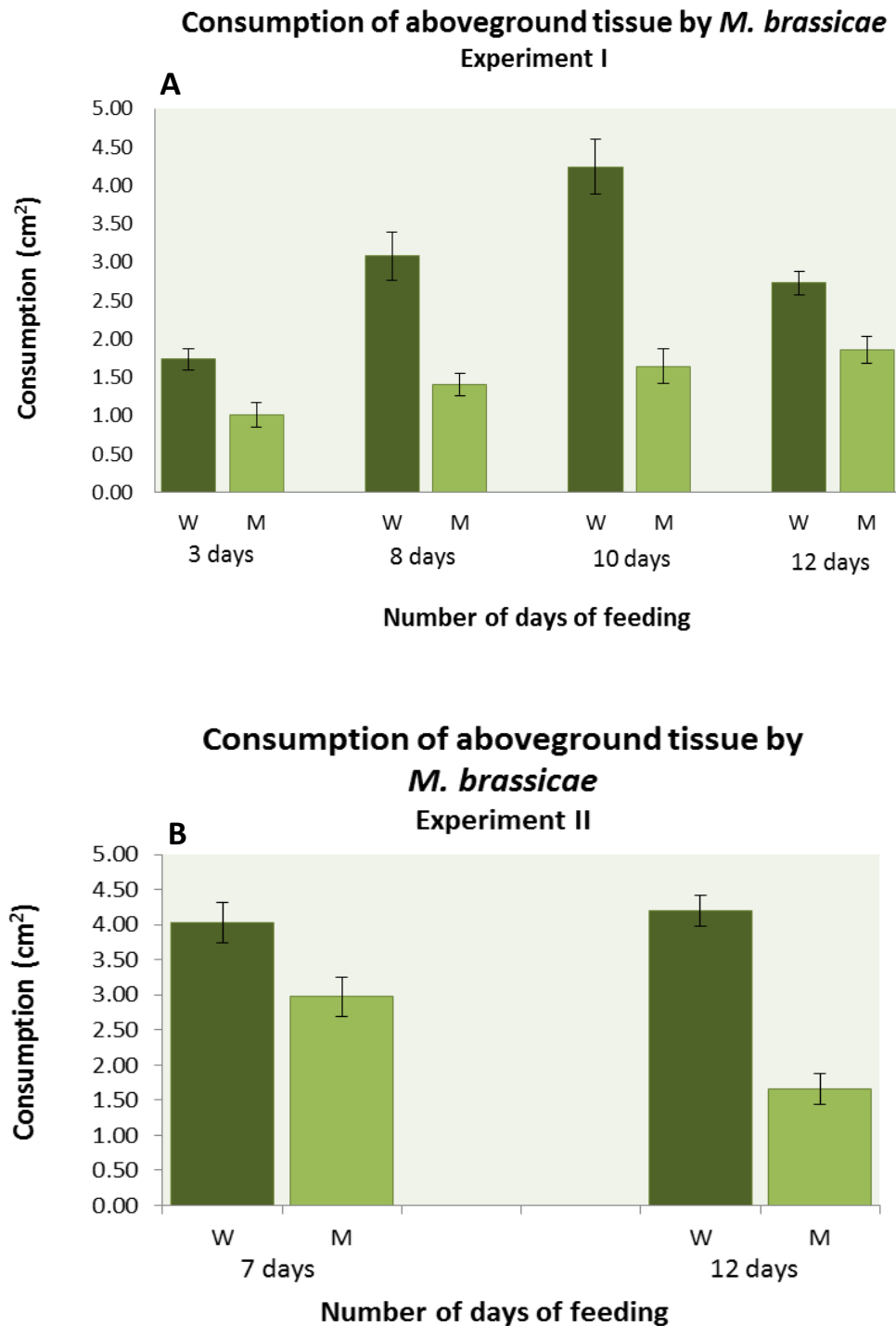
Figure 3.2.4: No-choice experiments. W-wild type, M-MINELESS.

***3.2.2.2 Insect weight experiments.*** Insect weight experiments after larval feeding show remarkable changes (Figure 3.2.5). Maximum weight gained by larvae was observed after 12 days feeding on aboveground tissues of wild-type plants in experiment I. Compared to 10 days feeding, after 12 days feeding larvae gained 3.5 times more weight. There was about 15-fold

increase on larval weight of 12 days feeding when compared to that of 8 days feeding, and 126-fold increase when compared to that of 3 days feeding (Figure 3.2.5 A).

After feeding on tissues of *MINELESS* plant, again larval maximum weight gain was observed on 12 days. Increase in number of days of feeding led to increase in larval weight. In all four time points of larval weight gain as a result of feeding on wild-type aboveground tissues was higher than that of *MINELESS*. Ten days feeding experiment showed the highest difference, where four-fold increase was observed after feeding on wild-type tissues compared to *MINELESS* tissues.
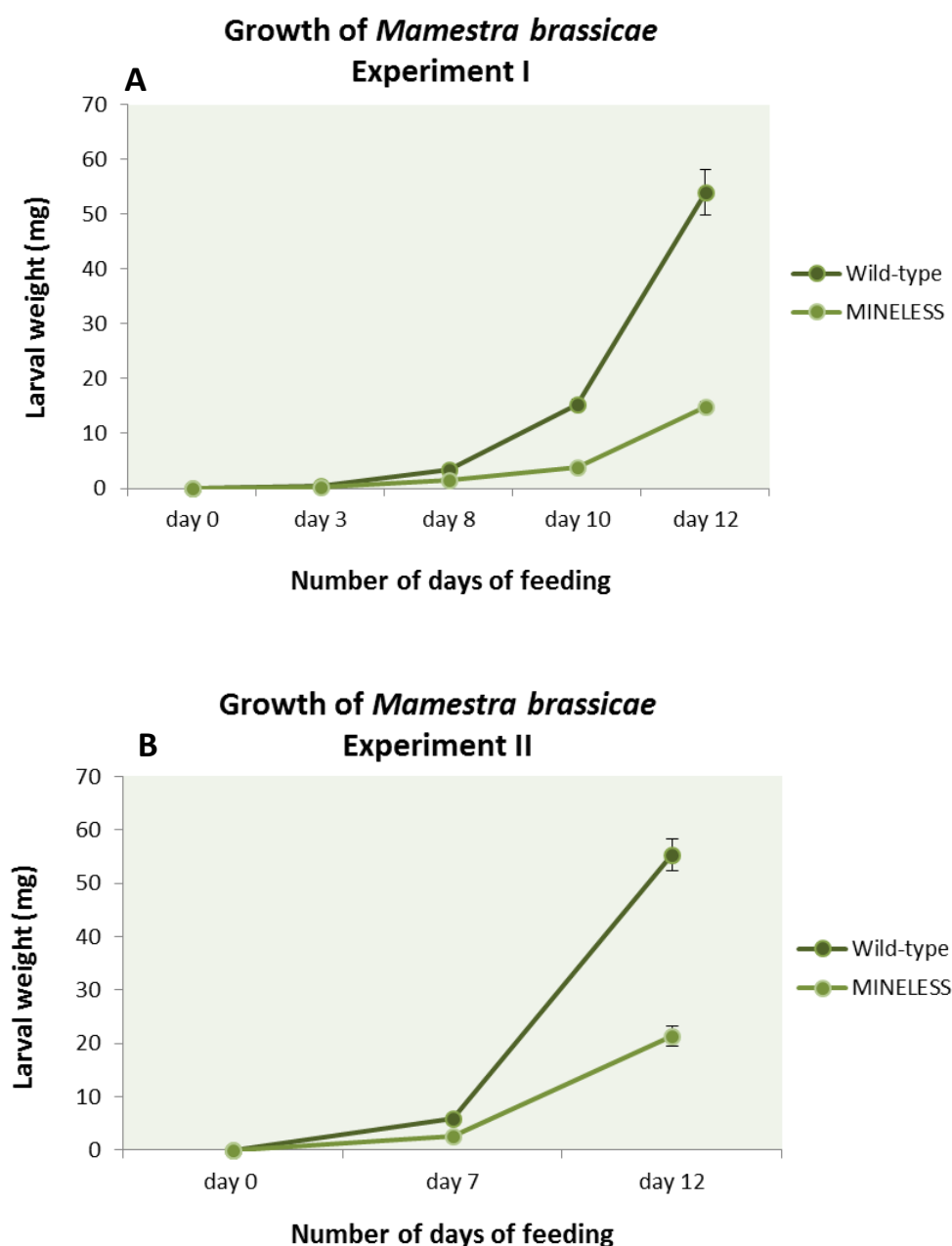


*Figure 3.2.5: No-choice experiments.*

In second set of experiment, weight gain obtained from 7 days and 12 days of larval feeding was introduced. Again an increase on feeding days led to increase in larval weight, which was 9-fold increase as a result of feeding on wild-type, and 8-fold increase after feeding on *MINELESS* aboveground tissues at the end of 12 days feeding experiment compared to that of 7 days. Both 7 days and 12 days feeding led to more weight gain in larvae fed on wild-type than that of on *MINELESS*, which about 2- and 3-fold weight increase were observed respectively (Figure 3.2.5 B).

## 3.3 Pollen beetles feeding and olfactometer experiments

### 3.3.1 Feeding experiment I

When racemes were infested and enclosed for feeding damage, the yellow buds, which are close to opening were not removed from the raceme (Fig 2.4.3). Therefore, during the 24 hours of the feeding experiment, those buds opened. Pollen beetles could freely feed from these buds, which would reduce the amount of damage caused to buds to feed. However, buds that had been damaged by feeding before opening could be determined by damage to the sepals and/or petals (Figure 3.3.1).
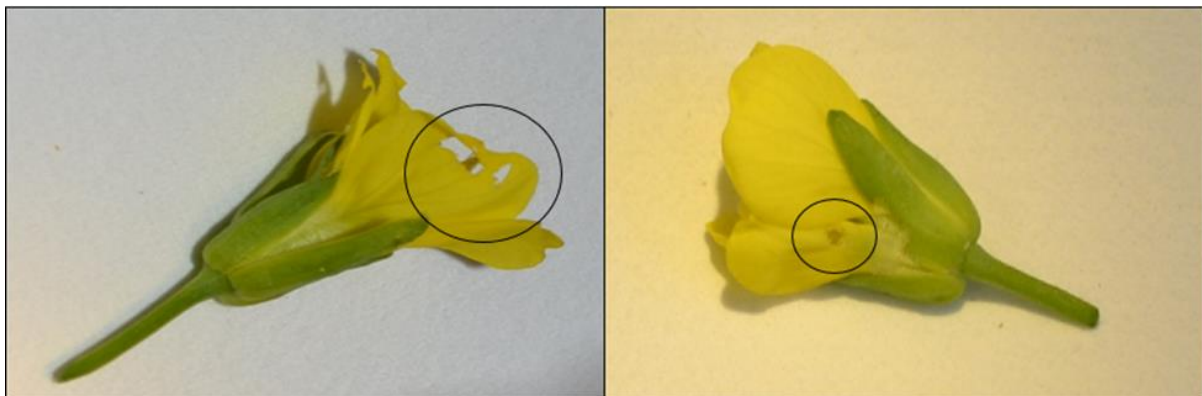


*Figure 3.3.1: Pollen beetle, M. aeneus, feeding damage on newly emerged petals from buds of B. napus in Feeding exp. I.*

In this experiment there was more feeding damage caused by pollen beetles assessed in wild-type buds than those in *MINELESS* (Figure 3.3.2). However, the difference was not significant (wild-type vs *MINELESS*: $\chi^2_1 = 0.01$; p = 0.92). There was no effect of previous plant damage to the plant damaged vs undamaged ($\chi^2_1 = 0.31$; p = 0.577) and there was no interaction ($\chi^2_1 = 2.52$; p = 0.113).
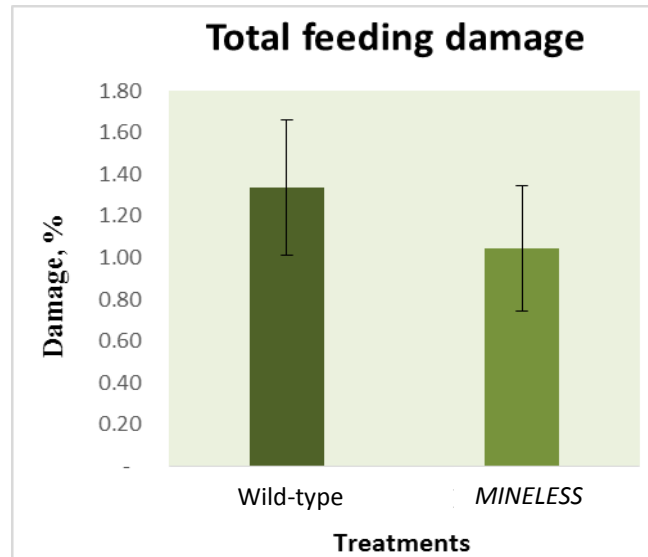
*Figure 3.3.2: Feeding Experiment I. Pollen beetle, M. aeneus, feeding damage on wild-type and MINELESS buds of B. napus. Values (±SE) are the mean of all treatments in overall replicas.*

***3.3.1.1 Sex determination.*** Excluding few damaged and missing pollen beetles, alltogether, 56 males and 51 females were identified in experiment 1.
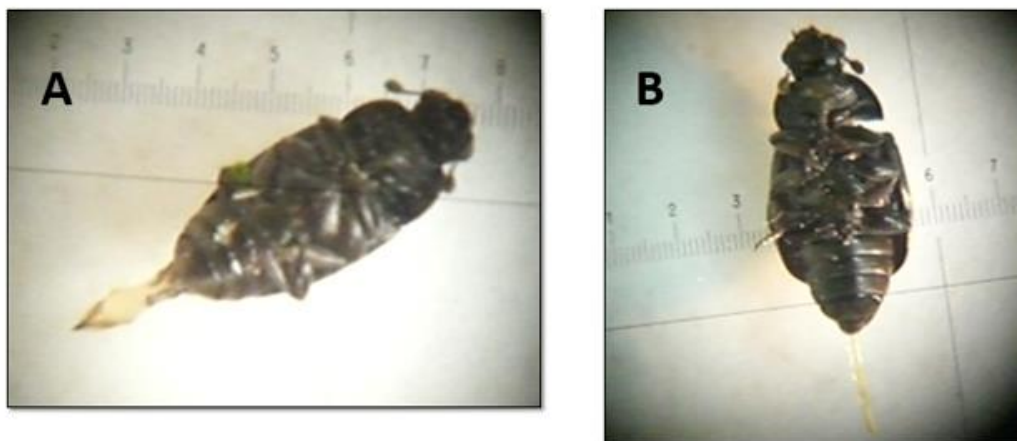


*Figure 3.3.3: Sex determination of pollen beetles, M. aeneus. A) Female ovipositor; B) Male aedegus (scale shows cm).*

## 3.3.2  Feeding experiment II

In this experiment, yellow buds were removed from buds and no flowers opened during the feeding period, thus enabling a fuller assessment of all feeding activity compared with Experiment I. As a result the proportion of buds damaged (Figure 3.3.4) was slightly higher than in Experiment I, but still quite low (Figure 3.3.5).
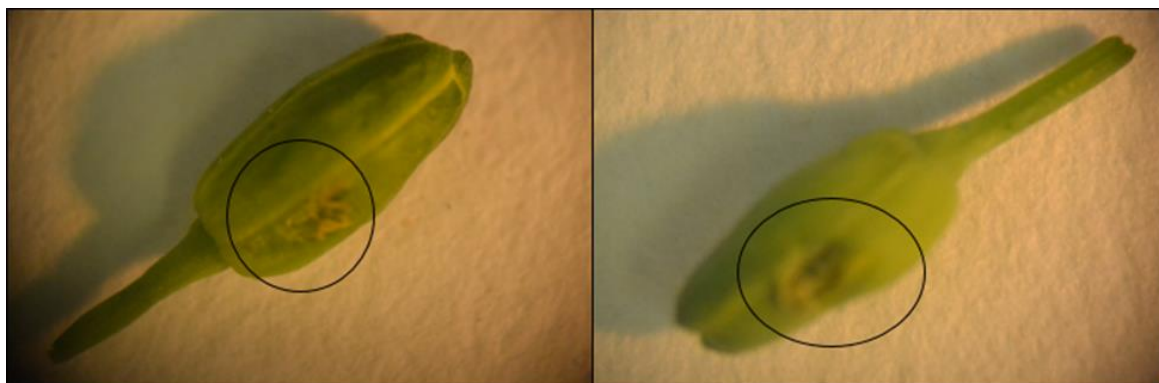
*Figure 3.3.4: Pollen beetle, M. aeneus, feeding damage on buds of B. napus in Experiment II.*

As in Experiment I, gain more feeding damage was observed to assessed in wild-type buds than those in of *MINELESS* plants;, there was 1.3 times more damage in wild-type buds however the difference was not significant χ2 with 1 d.f. = 1.15; p = 0.283.
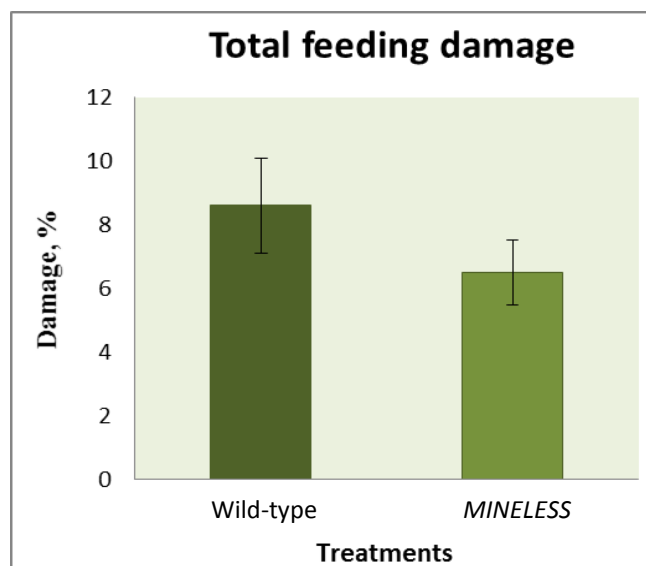


*Figure 3.3.5: Experiment 2. Pollen beetles, M. aeneus, feeding damage in wild-type and MINELESS buds of B. napus. Values (±SE) are the mean of all treatments in overall replicas.*

### 3.3.3 Y-tube-olfactometer

Oilseed rape odour versus control. Three beetles out of 18 were non-responsive; they either did not move to any direction, were inactive, or moved back to leave Y-tube. Overall 11 beetles orientated towards the oilseed rape odour and 4 beetles moved towards the control clean air control (Table 3.3.1). However, there was no significant difference (Fisher's Exact Test P=0.119).

*Table 3.3.1. Contingency table.*

| Response | OSR Odour L | OSR Odour R |
|----------|-------------|-------------|
| Left     | 7           | 3           |
| Right    | 1           | 4           |

# Chapter

## Discussion 4

## 4.1 Glucosinolate analysis from *Delia radicum* and *D. floralis* challenged material of *B. napus* wild-type and *MINELESS* plants

Four glucosinolates were detected in our study with the trace amount of other glucosinolates. Three of the four mainly detected glucosinolates were indole (indol-3-ylmethyl- (I3M); 4-methoxyindol-3-ylmethyl- (4MOI3M); 1-methoxyindol-3-ylmethyl- (1MOI3M)), and one of them was an aliphatic glucosinolate, 5-methylsulphinylpentyl- (5MSOP).

The amount of I3M glucosinolate was the highest among all detected glucosinolates in *B. napus* aboveground tissues. Previous studies also shows that I3M glucosinolate is the dominant indole glucosinolate in the leaves and shoots (Sang et al., 1984; Bodnaryk, 1992; Dam et al., 2009). Other cultivated species of Brassicaceae, especially *B. rapa* also exhibited the highest proportion of I3M glucosinolate in all tissues at all growth stages monitored (Bellostas et al., 2007). These high levels of indole glucosinolates, especially, I3M glucosinolate may indicate that some hydrolytic products are required for specific situations or developmental stages.

Unlike I3M glucosinolate, two other detected indole glucosinolates, 4MOI3M and 1MOI3M were in lower amounts. Earlier studies may explain this result by defined distribution patterns of indole glucosinolates; mostly I3M glucosinolate dominates in the leaves and 4MOI3M and 1MOI3M glucosinolates dominate in the hypocotyl and root (Sang et al., 1984; Giamoustaris and Mithen, 1995; Dam et al., 2009). One of the other cultivated species of Brassicaceae, five different varieties of *B. oleracea* showed high levels of I3M and low levels of 1MOI3M and 4MOI3M glucosinolates in aboveground tissues (Bellostas et al., 2007).

There was a slight difference on detected total glucosinolates and I3M glucosinolate levels between control wild-type and *MINELESS* aboveground tissues; *MINELESS* had slightly more I3M glucosinolate levels than wild-type. Obtained result agrees with several previous studies (Ahuja et al., 2011; Borgen et al., 2012). However, in another study, the detected levels of glucosinolates in leaves of undamaged cabbage plants, *B. oleracea*, were significantly higher in plants from the wild population than from the domesticated populations (Gols et al., 2008a).

Difference may be caused by different plant material analysed in these studies. However, the total glucosinolate levels was slightly higher in 4 days *MINELESS* treatment plants, in 10 days wild-type treatment plants, and was almost the same amount in 14 days *MINELESS* and wild-type plants. This result did not show exact difference between plant treatments.

Stress factors tend to increase glucosinolate content, presumably because of the increased amino acid and carbohydrate pools necessary for glucosinolate biosynthesis (Fenwick and Heaney, 1983). Glucosinolate concentrations in the shoots may rise after feeding by root herbivores. These root herbivores induced systemic responses have an influence on the behavior of aboveground specialist herbivores and also higher trophic levels connected with the same host plant (Hopkins et al., 2009). Indole glucosinolates slightly increased in four days challenged aboveground tissues. The levels of I3M glucosinolate both in control and challenged tissues were the highest among other detected glucosinolates. Increasing number of days of wounding by insects increased levels of indole glucosinolates compared to control tissues. Especially, ten days *D. radicum* challenged wild-type tissues showed the highest increase in all three detected indole glucosinolates compared to control tissues. I3M glucosinolates levels increased upon both herbivore feeding in all treatment days. Indole glucosinolates, e.g., may be a sink for production of the plant hormone indoleacetic acid (IAA) and thereby be involved in growth regulation (Bones et al., 1996). Thus, upon insect attack synthesis of indole glucosinolates increase may sustain further development of plant. Increased levels of indole glucosinolate in the cotyledons of one-week-old oilseed rape seedlings, *B. napus*, *B. rapa* , the mustard *B. juncea* after wounded mechanically or feeding by the specialist flea beetle, *P. cruciferae*, were also previously reported (Bodnaryk, 1992).

Detected aliphatic glucosinolate, 5MSOP, levels decreased in fourteen days insect challenged aboveground tissues compared to control tissues, however both control and insect challenged tissues showed higher levels of 5MSOP than those detected in 4 and 10 days of treatments. At the same time I3M glucosinolate levels increased upon insect herbivory, but levels of I3M was detected in lower amounts compared to those detected in 4 and 10 days of treatment. Plants often diminish their aliphatic and aromatic glucosinolates concentrations as a result of specialist than generalist herbivory (Koritsas et al., 1991; Hopkins et al., 1998; Dam and Raaijmakers, 2006). For instance, in *B. nigra*, aliphatic or aromatic glucosinolates decreased after feeding by *D. floralis* and *D. radicum*, while the same herbivory led to the increase of indolic glucosinolates in *B. oleracea* and *B. nigra* (Dam and Raaijmakers, 2006). During mechanical wounding indole glucosinolates, I3M and 1MOI3M, increased greatly. Such

changes were usually associated with large decreases in the levels of aliphatic glucosinolates. Thus increasing wounding was associated with an increase in indole glucosinolates and a decrease in aliphatic compounds (Bodnaryk, 1992) . These earlier studies correlates with our results.

Furthermore, overall levels of I3M glucosinolate both in wild-type and *MINELESS* control and insect challenged aboveground tissues decreased by increasing the number of days of insect infestation. However, 5MSOP glucosinolate levels mostly increased by increasing number of days of insect infestation. This result does not agree with number of previously reported studies. The selective catabolism of certain glucosinolates has been previously observed in other species belonging to the order Capparales. Bellostas et al. (2007) mentioned decrease in aliphatic glucosinolates levels with growth as the concentration of I3M glucosinolate increased in ripe seeds and sprouts of five varieties of *B. oleracea*. In leaves of *A. thaliana* the proportion of aliphatic glucosinolates declined with age resulting in the predominance of indole glucosinolates, mostly I3M glucosinolate and its 1-methoxyl derivative, by the time of senescence (Brown et al., 2003). During the first 7 days of imbibition of *B. napus* seeds, the concentration of the glucosinolates, mainly of the aliphatic type, decreases dramatically. At the same time, there is de novo synthesis of indole glucosinolates and 2-phenylethyl, 2PE (Chen and Andreasson, 2001). This may be the reason why mature tissues tend to have higher concentrations of indol-3-ylmethyl glucosinolates than young tissues. According to Clossais-Besnard et al. (1991), fluctuations in the amount and structure of the glucosinolates in life cycle suggest a storage function for these compounds which are required at specific stages of development. The different result obtained in our study could be explained by different plant material used in different studies. Oilseed rape cultivars have a restricted and uniform aliphatic glucosinolate profile, consisting of only butenyl and pentenyl glucosinolates and their hydroxylated homologues, although low levels of methylsulphinylpentyl glucosinolate are occasionally present (Magrath et al., 1994; Giamoustaris and Mithen, 1995) . It can be assumed that because of their actual low levels in plant tissues, aliphatic glucosinolates will not be detected in high levels like other glucosinolates even after herbivory.

The plant material that was used in our experiment contained aboveground parts of *B. napus,* which included shoots and leaves. The small part of homogenized aboveground tissue was used for extraction, and this tissue composition may vary among different biological replicas. For instance, some of them may contain more shoots than leaves, or opposite. This may also affect the different content of glucosinolates that were detected in tissues. Springett et al. (1989)

investigated the distribution of myrosinase in *B. oleracea* (Brussels sprouts) and concluded that myrosinase activity was 4-5 times higher in the outer leaves of the Brussels sprouts than in other parts, and the inner leaves of the rosette had much higher glucosinolate concentrations than the outer ones (Brown et al., 2003) . This shows that even in a single leaf myrosinase activity and glucosinolate content differs among different parts of the leaf. To avoid any unambiguity in obtained results, it would be better to use more homogenized plant material, such as extract from only leaves or particular parts of the stem, for the glucosinolate extraction and analysis.

## 4.2 *Mamestra Brassicae* feeding on *Brassica napus* cotyledons

In both no-choice and free choice experiments, *M. brassicae* larvae consumed more wild-type cotyledons than those of *MINELESS*. For generalist herbivores glucosinolates are exhibiting defensive features, however plays a role in host plant recognition for feeding and oviposition by specialist herbivores (Fahey et al., 2001). Generally glucosinolates demonstrate an adverse effects on generalist insect herbivores. In this way glucosinolates acts both as deterrents and attractants towards herbivores. (Hopkins et al., 2009). According to the study by Ahuja et al. (2011), in *MINELESS* seeds all the detected glucosinolates were significantly higher concentration than in the wild-type. Because *MINELESS* plants contain more glucosinolates than the wild-type plants and the protective properties of glucosinolates deterred feeding by larvae. This result correlates with number of studies. For instance, feeding on flower tissues of *B. nigra* with higher glucosinolate concentrations than leaves, provides specialist *Pieris brassicae* with a nutritional benefit in terms of higher growth rate (Smallegange et al., 2007), however specific foliar glucosinolates, such as sinigrin, which is predominant in *B. juncea*, and sinalbin, which is abundant in *S. alba*, may provide Brassicaceous crops with some protection from generalist *Mamestra configurata* (Ulmer et al., 2001). Herbivory by larvae of *M. brassicae* led to higher damage levels in the *myb28myb29* double mutant, Arabidopsis genotype without any aliphatic glucosinolates, compared to the Col-0 wild type, however both single mutants, *MYB28* and *MYB29*, had intermediate damage levels (Beekwilder et al., 2008). *M. brassicae* larvae preferred to feed and performed best on *Barbarea vulgaris* chemotype dominated by gluconasturtiin (NAS-type) than on that of dominated by glucobarbarin (BAR-type). BAR-type leaves had higher total glucosinolate levels than NAS-type leaves (van Leur et al., 2008). Larvae of the bertha armyworm*, M. configurata* fed young seedlings of *S. alba* performed poorly compared to larvae fed young seedlings of *B. napus* or older plants of both species. The high levels of sinalbin found in young cotyledons and leaves deterred the feeding

of the larvae of the *M. configurata* (Bodnaryk, 1991). McCloskey and Isman (1993) investigated the relationship between host plant glucosinolate profile and feeding and growth of, *M. configurata* by using eight cultivated rape and mustard varieties. Within *B. napus*, the numbers of larvae on the high-glucosinolate cultivar Midas did not differ significantly from the low-glucosinolate cultivars, Regent and Westar. However, with stage 3 foliage, significantly more larvae were attracted by Westar than by Regent. Although some of the above mentioned studies were carried out with different plant species from Brassicaceae family and with different herbivores, but all of them mention deterring features of glucosinolates against generalist herbivores.

Also it was reported that *MINELESS* plants are slower in development, and they show reduction in the main stem fresh and dry matter (Ahuja et al., 2011). This may be another factor affects the preference of wild-type cotyledons over *MINELESS* cotyledons by *M. brassicae* larvae.

In both no-choice experiments consumed area of cotyledons increased in correlation with increasing number of days of feeding, except 12 days feeding experiment. Ten days feeding resulted in maximum, and 3 days feeding experiment resulted in minimum wild-type cotyledon consumption in experiment I. It is possible that, larvae may reduce their activity because of developmental stages during 12 days feeding experiment. Cartea et al. (2014) showed that, larval weight increased quickly in the first few days as a result of leaf feeding of *M. brassicae* larvae in six cabbage varieties (*B. oleracea*), but increased slowly or did not increase at all thereafter as larvae entered the pre-pupal stage and therefore, reduced their activity.

Although *MINELESS* plants contain higher glucosinolates levels, increasing number of days of feeding resulted in slight increase in *MINELESS* cotyledon consumption in experiment I. An interaction between plant age and glucosinolates also may affect the development time of insect pests because glucosinolates are more concentrated in young plants than in mature plants and decline with plant age (Cartea et al., 2009).

No-choice experiments resulted in increase in larval weight with increase in number of days of feeding in both wild-type and *MINELESS* cotyledons. Both experiments resulted in higher larval weight when *M. brassicae* larvae fed on wild-type than in *MINELESS* cotyledons. Again, higher concentrations of glucosinolates in *MINELESS* plants may reduce larval feeding (Fahey et al., 2001; Hopkins et al., 2009; Ahuja et al., 2011), and as a result weight gain of larvae was less when feeding on *MINELESS* cotyledons compared to those of wild-type. There are number of previous studies which correlates with our results. On day 14, the average weight of *M.*

*brassicae* larvae raised on wild type plants was two to three times lower than on leaves from knock-out plants of Arabidopsis genotype without any aliphatic glucosinolates (Beekwilder et al., 2008). Study by Gols et al. (2008a) shows that, adult body mass and survival of the generalist herbivore, *M. brassicae* were significantly lower when reared on the wild *B. oleracea* strain than on cultivated population of cabbage, *B. oleracea*. The percentage of *M. brassicae* larvae that developed successfully into adults was highest on the cultivated population, slightly lower on the feral population, and the smallest on the wild population. Development and biomass of *M. brassicae* was similar on both cultivated and one wild cabbage, *B. oleracea* population (KIM), intermediate on the wild OH population, and significantly lower on the wild WIN population. Analysis of glucosinolates in leaves of the cabbages revealed higher levels in the wild populations than cultivars, with the highest concentrations in WIN plants (Harvey and Gols, 2011). Larvae of *M. brassicae* grew exponentially and had 100% survival on *Barbarea vulgaris* NAS-type leaves (dominated by gluconasturtiin), but hardly grew and had a high mortality when feeding on BAR-type leaves (dominated by glucobarbarin), which had on average higher total glucosinolate levels than NAS-type leaves (van Leur et al., 2008). Some other relevant studies also demonstrate low weight gain of generalist herbivores as a result of deterring effects of glucosinolates (McCloskey and Isman, 1993; Burow et al., 2006; Müller and Sieling, 2006).

## 4.3 Pollen beetle feeding and olfactometer experiments

We found no effect of the ablation of myrosinase in both feeding experiments by pollen beetles.

In experiment I, when racemes was infested with pollen beetles and enclosed with bread bags, there were yellow buds which were nearly flowered. Within twenty four hours feeding experiment those buds were replaced with flowers. To reach the pollen in the anthers inside the buds, the beetles must chew through the perianth and petals. After flowering beetles would feed from the pollen in anthers from open flowers and cause no more damage to buds to reach pollen (Williams and Free, 1978).

Several buds of the plants were mechanically damaged prior to feeding experiment I and other was remained intact. Mechanical damage was applied to induce plants and cause emission of defensive glucosinolate hydrolysis products. Generally glucosinolates exhibit an adverse effects on generalist insect herbivores (Hopkins et al., 2009). Pollen beetles, are specialist herbivores for oviposition but more generalist for feeding (Williams, 2010) and glucosinolates plays a role in host plant recognition for feeding and oviposition by specialist herbivores

(Bartlet et al., 1999; Fahey et al., 2001; Hopkins et al., 2009; Li et al., 2000). Pollen beetles are attracted to glucosinolate hydolysis products, especially to ITCs (Blight and Smart, 1999; Smart and Blight, 2000; Cook et al., 2006a). *MINELESS* plants as a result of ablation of myrosin cells should show very low and stable myrosinase activity, high glucosinolate concentrations, but low concentrations of glucosinolate hydrolysis products, including ITCs (Ahuja et al., 2011). Even high release rates of ITCs remain attractive to pollen beetles, in contrast to other insect species where ITCs become repellent (or inactive) when an upper limit is reached (Pivnick, 1993). For this reason it was expected that *MINELESS* oilseed rape plants will be less atrractive to pollen beetles than wild type plants. However, there was not significant difference between feeding on both damaged and undamaged wild-type and *MINELESS* plants. Detecting the concentrations and contents of glucosinolates and their hydrolysis products were not the aim of our study, but a possible explanation for these insignificant feeding results could be the low glucosinolate and ITCs emission by damaged buds. In both conducted feeding experiments, most of the time beetles chewed sepals or petals to reach to pollen, and Giamoustaris and Mithen (1996) showed that, it is likely that ITCs released after biting may be mildly repellent or deterrent. This could give the reason why beetles did not exhibit significant damage to wild-type buds than those to *MINELESS*. At full flowering stage the beetles can feed directly on the pollen without having to damage any tissues and thus may avoid glucosinolates and their hydrolytic products. However, the deterrence conferred by glucosinolates is not very effective as it only operates prior to flower opening (Giamoustaris and Mithen, 1996). Furthermore, it may also possible that there were another volatiles present as a result of feeding by beetles, and it might affected feeding behavior of beetles. Smart and Blight (2000) showed that, within the release rate range of 5-30 mg/day, *M. aeneus* may respond nonspecifically to all, or at least a large number of, ITCs. In this, the beetle differs from other brassica-associated insects. MINELESS plants are slow in development, large reduction in the main stem fresh and dry matter was observed (Ahuja et al., 2011). These factors may affect feeding behaviour of insects. However, *MINELESS* plants used in experiment 1 and 2 showed proper growth and development as wild-type plants did.

In the Y-tube olfactometer experiments, more pollen beetles moved towards the arm into which the odour of oilseed rape racemes was introduced than to the control; however the difference was not significant. This was probably due to the small number of individuals tested. In previous studies it has been shown that pollen beetles are attracted to the odour of oilseed rape (Cook et al., 2002). Oilseed rape leaf and flower odour was attractive to pollen beetles when

was observed in the laboratory using a fourarmed airflow olfactometer, and under semi-natural conditions using a markrelease–recapture technique (Evans and Allen-Williams, 1994).

## 4.4  Future study

The result obtained from glucosinolates extraction and analysis in Xevo$^{TM}$ TQ-S from control and insect challenged wild-type and *MINELESS B. napus* aboveground tissues is in itself new and will require more such kind of experiments with tissues from different separate organs of plant rather than a mixture of aboveground tissues. Also insect challenged days can be increased to assess exact difference between insect-induced and non-induced plants. Pollen beetles feeding and olfactometer experiments did not show significant results, therefore it is hard to assess, however in order to gain more precise information in this respect future studies should aim at using high number of pollen beetles in feeding and olfactometer experiments.

# Chapter 5

# Conclusion

Glucosinolates from control and *Delia radicum* and *D. floralis* challenged plant materials were analysed in Xevo$^{TM}$ TQ-S (Tandem Quadrupole Spectrometer with Stepwave ion guide). According to the results of this study, indol-3-ylmethyl- (I3M) glucosinolate was the most abundant glucosinolate both in control and insect challenged aboveground tissues of *B. napus* plants. In all treatments I3M levels increased upon insect feeding, and slightly higher concentrations of I3M glucosinolates were detected in control and insect challenged *MINELESS* plants than wild-type plants. Other indole glucosinolates, 4-methoxyindol-3-ylmethyl- (4MOI3M) and 1-methoxyindol-3-ylmethyl- (1MOI3M) were detected in a lower amounts and did not show exact difference between *MINELESS* and wild-type plants and also after herbivory. Increasing days of treatment increased levels of aliphatic glucosinolate, 5-methylsulphinylpentyl- (5MSOP), and decreased levels of indole glucosinolate, I3M glucosinolates. In both no-choice and free-choice experiments, *M. brassicae* larvae preferred to feed on more wild-type cotyledons than those of *MINELESS*, and therefore more area in wild-type cotyledons was damaged after feeding by larvae. Pollen beetles fed on more in wild-type buds, whereas there was not a significant difference on feeding damage between wild-type and *MINELESS* buds of *B. napus* plants. Due to the small number of individuals tested, we did not found significant difference in Y-tube olfactometer experiment, however more beetles preferred oilseed rape flower odour over fresh air on their movements.

# Chapter

# References 6

AHUJA, I., BORGEN, B. H., HANSEN, M., HONNE, B. I., MÜLLER, C., ROHLOFF, J., ROSSITER, J. T. & BONES, A. M. 2011. Oilseed rape seeds with ablated defence cells of the glucosinolate–myrosinase system. Production and characteristics of double haploid MINELESS plants of Brassica napus L. *Journal of Experimental Botany,* 62**,** 4975-4993.

AHUJA, I., ROHLOFF, J. & BONES, A. 2010. Defence mechanisms of Brassicaceae: implications for plant-insect interactions and potential for integrated pest management. A review. *Agronomy for Sustainable Development,* 30**,** 311-348.

ALFORD, D. V., NILSSON, C. & ULBER, B. 2003. Insect pests of oilseed rape crops. *Biocontrol of oilseed rape pests***,** 9-41.

BAKR, E. M. 2005. A new software for measuring leaf area, and area damaged by Tetranychus urticae Koch. *Journal of Applied Entomology,* 129**,** 173-175.

BARTH, C. & JANDER, G. 2006. Arabidopsis myrosinases TGG1 and TGG2 have redundant function in glucosinolate breakdown and insect defense. *The Plant Journal,* 46**,** 549-562.

BARTLET, E., KIDDLE, G., WILLIAMS, I. & WALLSGROVE, R. 1999. Wound-induced increases in the glucosinolate content of oilseed rape and their effect on subsequent herbivory by a crucifer specialist. *In:* SIMPSON, S., MORDUE, A. J. & HARDIE, J. (eds.) *Proceedings of the 10th International Symposium on Insect-Plant Relationships.* Springer Netherlands.

BEEKWILDER, J., VAN LEEUWEN, W., VAN DAM, N. M., BERTOSSI, M., GRANDI, V., MIZZI, L., SOLOVIEV, M., SZABADOS, L., MOLTHOFF, J. W., SCHIPPER, B., VERBOCHT, H., DE VOS, R. C. H., MORANDINI, P., AARTS, M. G. M. & BOVY, A. 2008. The Impact of the Absence of Aliphatic Glucosinolates on Insect Herbivory in Arabidopsis. *PLoS ONE,* 3**,** e2068.

BELLOSTAS, N., KACHLICKI, P., SØRENSEN, J. C. & SØRENSEN, H. 2007. Glucosinolate profiling of seeds and sprouts of B. oleracea varieties used for food. *Scientia Horticulturae,* 114**,** 234-242.

BIRCH, A. N. E., GRIFFITHS, D. W., HOPKINS, R. J. & SMITH, W. H. M. 1996. A time-course study of chemical and physiological responses in Brassicas induced by turnip root fly (Delia floralis) larval feeding. *Entomologia Experimentalis et Applicata,* 80**,** 221-223.

BJÖRKMAN, M., KLINGEN, I., BIRCH, A. N. E., BONES, A. M., BRUCE, T. J. A., JOHANSEN, T. J., MEADOW, R., MØLMANN, J., SELJÅSEN, R., SMART, L. E. & STEWART, D. 2011. Phytochemicals of Brassicaceae in plant protection and human health – Influences of climate, environment and agronomic practice. *Phytochemistry,* 72**,** 538-556.

BLIGHT, M. & SMART, L. 1999. Influence of Visual Cues and Isothiocyanate Lures on Capture of the Pollen Beetle, Meligethes aeneus in Field Traps. *Journal of Chemical Ecology,* 25**,** 1501-1516.

BLOSSEY, B. & HUNT-JOSHI, T. R. 2003. BELOWGROUND HERBIVORY BY INSECTS: Influence on Plants and Aboveground Herbivores. *Annual Review of Entomology,* 48**,** 521-547.

BODNARYK, R. P. 1991. Developmental profile of sinalbin (p-hydroxybenzyl glucosinolate) in mustard seedlings, Sinapis alba L., and its relationship to insect resistance. *Journal of chemical ecology,* 17**,** 1543-1556.

BODNARYK, R. P. 1992. Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard. *Phytochemistry,* 31**,** 2671-2677.

BONES, A. M. & ROSSITER, J. T. 1996. The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiologia Plantarum,* 97**,** 194-208.

BONES, A. M. & ROSSITER, J. T. 2006. The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry,* 67**,** 1053-1067.

BORGEN, B. H., AHUJA, I., THANGSTAD, O. P., HONNE, B. I., ROHLOFF, J., ROSSITER, J. T. & BONES, A. M. 2012. 'Myrosin cells' are not a prerequisite for aphid feeding on oilseed rape (Brassica napus) but affect host plant preferences. *Plant Biology,* 14**,** 894-904.

BORGEN, B. H., THANGSTAD, O. P., AHUJA, I., ROSSITER, J. T. & BONES, A. M. 2010. Removing the mustard oil bomb from seeds: transgenic ablation of myrosin cells in oilseed rape (Brassica napus) produces MINELESS seeds. *Journal of Experimental Botany,* 61**,** 1683-1697.

BROWN, P. D., TOKUHISA, J. G., REICHELT, M. & GERSHENZON, J. 2003. Variation of glucosinolate accumulation among different organs and developmental stages of Arabidopsis thaliana. *Phytochemistry,* 62**,** 471-481.

BRUCE, T. J. A. 2014. Glucosinolates in oilseed rape: secondary metabolites that influence interactions with herbivores and their natural enemies. *Annals of Applied Biology,* 164, 348-353.

BUROW, M., MÜLLER, R., GERSHENZON, J. & WITTSTOCK, U. 2006. Altered Glucosinolate Hydrolysis in Genetically Engineered Arabidopsis thaliana and its Influence on the Larval Development of Spodoptera littoralis. *Journal of Chemical Ecology,* 32**,** 2333-2349.

CARTEA, M. E., FRANCISCO, M., LEMA, M., SOENGAS, P. & VELASCO, P. 2010. Resistance of Cabbage (Brassica oleracea capitata Group) Crops to Mamestra brassicae. *Journal of Economic Entomology,* 103**,** 1866-1874.

CARTEA, M. E., SOENGAS, P., ORDÁS, A. & VELASCO, P. 2009. Resistance of kale varieties to attack by Mamestra brassicae. *Agricultural and Forest Entomology,* 11**,** 153-160.

CARTEA, M. E., SOENGAS, P., SOTELO, T., ABILLEIRA, R. & VELASCO, P. 2014. Determining the host-plant resistance mechanisms for Mamestra brassicae (Lepidoptera: Noctuidae) pest in cabbage. *Annals of Applied Biology,* 164**,** 270-285.

CHEN, S. & ANDREASSON, E. 2001. Update on glucosinolate metabolism and transport. *Plant Physiology and Biochemistry,* 39**,** 743-758.

CLOSSAIS-BESNARD, N. & LARHER, F. 1991. Physiological role of glucosinolates in brassica napus. Concentration and distribution pattern of glucosinolates among plant organs during a complete life cycle. *Journal of the Science of Food and Agriculture,* 56**,** 25-38.

COOK, S. M., BARTLET, E., MURRAY, D. A. & WILLIAMS, I. H. 2002. The role of pollen odour in the attraction of pollen beetles to oilseed rape flowers. *Entomologia Experimentalis et Applicata,* 104**,** 43-50.

COOK, S. M., SMART, L. E., MARTIN, J. L., MURRAY, D. A., WATTS, N. P. & WILLIAMS, I. H. 2006a. Exploitation of host plant preferences in pest management strategies for oilseed rape (Brassica napus). *Entomologia Experimentalis et Applicata,* 119**,** 221-229.

COOK, S. M., WATTS, N. P., CASTLE, L. M. & WILLIAMS, I. H. 2006b. Determining the sex of insect pests of oilseed rape for behavioural bioassays. *IOBC WPRS BULLETIN,* 29**,** 205.

DAM, N., TYTGAT, T. G. & KIRKEGAARD, J. 2009. Root and shoot glucosinolates: a comparison of their diversity, function and interactions in natural and managed ecosystems. *Phytochemistry Reviews,* 8**,** 171-186.

DAM, N. M. V., WITJES, L. & SVATOŠ, A. 2004. Interactions between Aboveground and Belowground Induction of Glucosinolates in Two Wild Brassica Species. *New Phytologist,* 161**,** 801-810.

DAM, N. V. & RAAIJMAKERS, C. 2006. Local and systemic induced responses to cabbage root fly larvae (Delia radicum) in Brassica nigra and B. oleracea. *Chemoecology,* 16**,** 17-24.

DE JONG, R. & STÄDLER, E. 1999. The influence of odour on the oviposition behaviour of the cabbage root fly. *Chemoecology,* 9**,** 151-154.

DEVETAK, M., BOHINC, T. & TRDAN, S. 2013. Natural resistance of ten cabbage genotypes to cabbage moth (Mamestra brassicae [L.]) attack under field conditions. *International journal of food, agriculture and environment,* 11**,** 908-914.

DEVETAK, M., VIDRIH, M. & TRDAN, S. 2010. Cabbage moth (Mamestra brassicae [L.]) and bright-line brown-eyes moth (Mamestra oleracea [L.]) - presentation of the species, their monitoring and control measures. *Acta agriculturae Slovenica.*

DUPONT, J., WHITE, P. J., JOHNSTON, K. M., HEGGTVEIT, H. A., MCDONALD, B. E., GRUNDY, S. M. & BONANOME, A. 1989. Food safety and health effects of canola oil. *Journal of the American College of Nutrition,* 8**,** 360-375.

EKBOM, B. 2010. Pests and Their Enemies in Spring Oilseed Rape in Europe and Challenges to Integrated Pest Management. *In:* WILLIAMS, I. H. (ed.) *Biocontrol-Based Integrated Management of Oilseed Rape Pests.* Springer Netherlands.

EKBOM, B. & BORG, A. 1996. Pollen beetle (Meligethes aeneus) oviposition and feeding preference on different host plant species. *Entomologia Experimentalis et Applicata,* 78**,** 291-299.

EVANS, K. A. & ALLEN-WILLIAMS, L. J. 1994. Laboratory and field response of the pollen beetle, Meligethes aeneus, to the odour of oilseed rape. *Physiological Entomology,* 19**,** 285-290.

FAHEY, J. W., ZALCMANN, A. T. & TALALAY, P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry,* 56**,** 5-51.

FAO 2013. World Food and Agriculture. *FAO STATISTICAL YEARBOOK, Food and Agriculture Organization of the United Nations, Rome*.

FENWICK, G. R. & HEANEY, R. K. 1983. Glucosinolates and their breakdown products in cruciferous crops, foods and feedingstuffs. *Food Chemistry,* 11**,** 249-271.

GIAMOUSTARIS, A. & MITHEN, R. 1995. The effect of modifying the glucosinolate content of leaves of oilseed rape (Brassica napus ssp. oleifera) on its interaction with specialist and generalist pests. *Annals of Applied Biology,* 126**,** 347-363.

GIAMOUSTARIS, A. & MITHEN, R. 1996. The effect of flower colour and glucosinolates on the interaction between oilseed rape and pollen beetles. *In:* STÄDLER, E., ROWELL-RAHIER, M. & BAUER, R. (eds.) *Proceedings of the 9th International Symposium on Insect-Plant Relationships.* Springer Netherlands.

GLAUSER, G., SCHWEIZER, F., TURLINGS, T. C. J. & REYMOND, P. 2012. Rapid Profiling of Intact Glucosinolates in Arabidopsis Leaves by UHPLC-QTOFMS Using a Charged Surface Hybrid Column. *Phytochemical Analysis,* 23**,** 520-528.

GOLS, R., BUKOVINSZKY, T., DAM, N., DICKE, M., BULLOCK, J. & HARVEY, J. 2008a. Performance of Generalist and Specialist Herbivores and their Endoparasitoids Differs on Cultivated and Wild Brassica Populations. *Journal of Chemical Ecology,* 34**,** 132-143.

GOLS, R., WAGENAAR, R., BUKOVINSZKY, T., DAM, N. M. V., DICKE, M., BULLOCK, J. M. & HARVEY, J. A. 2008b. GENETIC VARIATION IN DEFENSE CHEMISTRY IN WILD CABBAGES AFFECTS HERBIVORES AND THEIR ENDOPARASITOIDS. *Ecology,* 89**,** 1616-1626.

GRUBB, C. D. & ABEL, S. 2006. Glucosinolate metabolism and its control. *Trends in Plant Science,* 11**,** 89-100.

HALKIER, B. A. & GERSHENZON, J. 2006. Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology,* 57**,** 303-333.

HARVEY, J. A. & GOLS, R. 2011. Population-related variation in plant defense more strongly affects survival of an herbivore than its solitary parasitoid wasp. *Journal of chemical ecology,* 37**,** 1081-1090.

HIIESAAR, K., METSPALU, L., LÄÄNISTE, P., JÕGAR, K., KUUSIK, A. & JÕUDU, J. 2003. Insect pests on winter oilseed rape studied by different catching methods. *Agronomy research,* 1**,** 17-29.

HOKKANEN, H. M. T. 2000. The making of a pest: recruitment of Meligethes aeneus onto oilseed Brassicas. *Entomologia Experimentalis et Applicata,* 95**,** 141-149.

HOPKINS, R., EKBOM, B. & HENKOW, L. 1998. Glucosinolate Content and Susceptibility for Insect Attack of Three Populations of Sinapis alba. *Journal of Chemical Ecology,* 24**,** 1203-1216.

HOPKINS, R. J., BIRCH, A. N. E., GRIFFITHS, D. W., BAUR, R., STÄDLER, E. & MCKINLAY, R. G. 1997. Leaf Surface Compounds and Oviposition Preference of Turnip Root Fly Delia floralis: The Role of Glucosinolate and Nonglucosinolate Compounds. *Journal of Chemical Ecology,* 23**,** 629-643.

HOPKINS, R. J., VAN DAM, N. M. & VAN LOON, J. J. A. 2009. Role of Glucosinolates in Insect-Plant Relationships and Multitrophic Interactions. *Annual Review of Entomology,* 54**,** 57-83.

JAKOBSEN, H. B., FRIIS, P., NIELSEN, J. K. & OLSEN, C. E. 1994. Emission of volatiles from flowers and leaves of Brassica napus in situ. *Phytochemistry,* 37**,** 695-699.

JOHANSEN, N. S. 1997. Mortality of eggs, larvae and pupae and larval dispersal of the cabbage moth, Mamestra brassicae, in white cabbage in south-eastern Norway. *Entomologia Experimentalis et Applicata,* 83**,** 347-360.

JÖNSSON, M. & ANDERSON, P. 2007. Emission of oilseed rape volatiles after pollen beetle infestation; behavioural and electrophysiological responses in the parasitoid Phradis morionellus. *Chemoecology,* 17**,** 201-207.

KLIEBENSTEIN, D., PEDERSEN, D., BARKER, B. & MITCHELL-OLDS, T. 2002. Comparative Analysis of Quantitative Trait Loci Controlling Glucosinolates, Myrosinase and Insect Resistance in Arabidopsis thaliana. *Genetics,* 161**,** 325-332.

KLINGEN, I., MEADOW, R. & AANDAL, T. 2002. Mortality of Delia floralis, Galleria mellonella and Mamestra brassicae treated with insect pathogenic hyphomycetous fungi. *Journal of Applied Entomology,* 126**,** 231-237.

KORITSAS, V. M., LEWIS, J. A. & FENWICK, G. R. 1991. Glucosinolate responses of oilseed rape, mustard and kale to mechanical wounding and infestation by cabbage stem flea beetle (Psylliodes chrysocephala). *Annals of Applied Biology,* 118**,** 209-221.

LAMB, R. J. 1989. Entomology of Oilseed Brassica Crops. *Annual Review of Entomology,* 34**,** 211-229.

LAMBRIX, V., REICHELT, M., MITCHELL-OLDS, T., KLIEBENSTEIN, D. J. & GERSHENZON, J. 2001. The Arabidopsis Epithiospecifier Protein Promotes the Hydrolysis of Glucosinolates to Nitriles and Influences Trichoplusia ni Herbivory. *The Plant Cell Online,* 13**,** 2793-2807.

LEATHER, S. R. 2014. Modifying glucosinolates in oilseed rape – Giamoustaris & Mithen (1995): a top-twenty paper in the Annals of Applied Biology. *Annals of Applied Biology,* 164**,** 318-319.

LI, Q., EIGENBRODE, S., STRINGAM, G. R. & THIAGARAJAH, M. R. 2000. Feeding and Growth of Plutella xylostella and Spodoptera eridania on Brassica juncea with Varying Glucosinolate Concentrations and Myrosinase Activities. *Journal of Chemical Ecology,* 26**,** 2401-2419.

MAGRATH, R., BANO, F., MORGNER, M., PARKIN, I., SHARPE, A., LISTER, C., . . . MITHEN, R. 1994. Genetics of aliphatic glucosinolates. i. side chain elongation in Brassica napus and Arabidopsis thaliana. Heredity, 72(3), 290-299.

MCCLOSKEY, C. & ISMAN, M. B. 1993. Influence of foliar glucosinolates in oilseed rape and mustard on feeding and growth of the bertha armyworm, Mamestra configurata Walker. *Journal of chemical ecology,* 19**,** 249-266.

MILFORD, G. F. J., PORTER, A. J. R., FIELDSEND, J. K., MILLER, C. A., LEACH, J. E. & WILLIAMS, I. H. 1989. Glucosinolates in oilseed rape (Brassica napus) and the incidence of pollen beetles (Meligethes aeneus). *Annals of Applied Biology,* 115**,** 375-380.

MÜLLER, C. & SIELING, N. 2006. Effects of glucosinolate and myrosinase levels in Brassica juncea on a glucosinolate-sequestering herbivore – and vice versa. *CHEMOECOLOGY,* 16**,** 191-201.

NILSSON, C. 1988. The number of larval instars of Meligethes aeneus (F.) in southern Sweden. *Växtskyddsnotiser* 52**,** 151-152.

OSBORNE, P. 1965. Morphology of the immature stages of Meligethes aeneus (F.) and M. viridescens (F.) (Coleoptera, Nitidulidae). *Bulletin of Entomological Research,* 55**,** 747-759.

PIVNICK, K. A. 1993. Response ofMeteorus leviventris,(Hymenoptera: Braconidae) to mustard oils in field trapping experiments. *Journal of chemical ecology,* 19**,** 2075-2079.

POELMAN, E. H., BROEKGAARDEN, C., VAN LOON, J. J. & DICKE, M. 2008. Early season herbivore differentially affects plant defence responses to subsequently colonizing herbivores and their abundance in the field. *Molecular Ecology,* 17**,** 3352-3365.

RAKOW, G. 2004. Species origin and economic importance of Brassica. *Brassica.* Springer.

RATZKA, A., VOGEL, H., KLIEBENSTEIN, D. J., MITCHELL-OLDS, T. & KROYMANN, J. 2002. Disarming the mustard oil bomb. *Proceedings of the National Academy of Sciences,* 99**,** 11223-11228.

ROESSINGH, P., STÄDLER, E., BAUR, R., HURTER, J. & RAMP, T. 1997. Tarsal chemoreceptors and oviposition behaviour of the cabbage root fly (Delia radicum) sensitive to fractions and new compounds of host-leaf surface extracts. *Physiological Entomology, 22*, 140-148.

ROJAS, WYATT & BIRCH 2001. Oviposition by Mamestra brassicae (L.) (Lep., Noctuidae) in relation to age, time of day and host plant. *Journal of Applied Entomology, 125*, 161-163.

ROJAS, J., WYATT, T. & BIRCH, M. 2000. Flight and Oviposition Behavior Toward Different Host Plant Species by the Cabbage Moth, Mamestra brassicae (L.) (Lepidoptera: Noctuidae). *Journal of Insect Behavior, 13*, 247-254.

RUTHER, J. & THIEMANN, K. 1997. Response of the pollen beetle Meligethes aeneus to volatiles emitted by intact plants and conspecifics. *Entomologia experimentalis et applicata, 84*, 183-188.

SANG, J. P., MINCHINTON, I. R., JOHNSTONE, P. K. & TRUSCOTT, R. J. W. 1984. Glucosinolate profiles in the seed, root and leaf tissue of cabbage, mustard, rapeseed, radish and swede. *Canadian Journal of Plant Science, 64*, 77-93.

SCHLAEPPI, K., BODENHAUSEN, N., BUCHALA, A., MAUCH, F. & REYMOND, P. 2008. The glutathione-deficient mutant pad2-1 accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore Spodoptera littoralis. *The Plant Journal, 55*, 774-786.

SELJÅSEN, R. & MEADOW, R. 2006. Effects of neem on oviposition and egg and larval development of Mamestra brassicae L: Dose response, residual activity, repellent effect and systemic activity in cabbage plants. *Crop Protection, 25*, 338-345.

SIEMENS, D. H., MITCHELL & OLDS, T. 1996. Glucosinolates and Herbivory by Specialists (Coleoptera: Chrysomelidae, Lepidoptera: Plutellidae): Consequences of Concentration and Induced Resistance. *Environmental Entomology, 25*, 1344-1353.

SINGH, B. P. 2013. *Biofuel crops: production, physiology and genetics*, CABI.

SMALLEGANGE, R. C., VAN LOON, J. J. A., BLATT, S. E., HARVEY, J. A., AGERBIRK, N. & DICKE, M. 2007. Flower vs. Leaf Feeding by Pieris brassicae: Glucosinolate-Rich Flower Tissues are Preferred and Sustain Higher Growth Rate. *Journal of Chemical Ecology, 33*, 1831-1844.

SMART, L. & BLIGHT, M. 2000. Response of the Pollen Beetle, Meligethes aeneus, to Traps Baited with Volatiles from Oilseed Rape, Brassica napus. *Journal of Chemical Ecology, 26*, 1051-1064.

SNOWDON, R., LÜHS, W. & FRIEDT, W. 2007. Oilseed Rape. *In:* KOLE, C. (ed.) *Oilseeds.* Springer Berlin Heidelberg.

SPRINGETT, M. B. & ADAMS, J. B. 1989. Properties of Brussels sprouts thioglucosidase. *Food Chemistry, 33*, 173-186.

STEFANSSON, B., HOUGEN, F. W. & DOWNEY, R. 1961. Note on the isolation of rape plants with seed oil free from erucic acid. *Canadian Journal of Plant Science, 41*, 218-219.

TATCHELL, G. M. 1983. Compensation in spring-sown oil-seed rape ( Brassica napus L.) plants in response to injury to their flower buds and pods. *The Journal of Agricultural Science, 101*, 565-573.

TEXTOR, S. & GERSHENZON, J. 2009. Herbivore induction of the glucosinolate–myrosinase defense system: major trends, biochemical bases and ecological significance. *Phytochemistry Reviews, 8*, 149-170.

TÖLLE, M. 2014. Factors regulating the population dynamics and damage potential of pollen beetle (Meligethes aeneus F.) on crops of oilseed rape, Georg-August-Universität Göttingen.

TRAUTWEIN, E. A. 1997. Food quality of rapeseed oil–aspects from a nutritional point of view. *GCIRC Bull, 14*, 123-128.

ULLAND, S., IAN, E., MOZURAITIS, R., BORG-KARLSON, A.-K., MEADOW, R. & MUSTAPARTA, H. 2008. Methyl Salicylate, Identified as Primary Odorant of a Specific Receptor Neuron Type, Inhibits Oviposition by the Moth Mamestra Brassicae L. (Lepidoptera, Noctuidae). *Chemical Senses, 33*, 35-46.

ULMER, B., GILLOTT, C. & ERLANDSON, M. 2001. Feeding preferences, growth, and development of Mamestra configurata (Lepidoptera: Noctuidae) on Brassicaceae. *The Canadian Entomologist,* 133**,** 509-519.

ULMER, B. J. & DOSDALL, L. M. 2006. Glucosinolate profile and oviposition behavior in relation to the susceptibilities of Brassicaceae to the cabbage seedpod weevil. *Entomologia Experimentalis et Applicata,* 121**,** 203-213.

VAN DAM, N. M., SAMUDRALA, D., HARREN, F. J. & CRISTESCU, S. M. 2012. Real-time analysis of sulfur-containing volatiles in Brassica plants infested with root-feeding Delia radicum larvae using proton-transfer reaction mass spectrometry. *AoB Plants,* 2012**,** pls021.

VAN LEUR, H., VET, L. M., VAN DER PUTTEN, W. & VAN DAM, N. 2008. Barbarea vulgaris Glucosinolate Phenotypes Differentially Affect Performance and Preference of Two Different Species of Lepidopteran Herbivores. *Journal of Chemical Ecology,* 34**,** 121-131.

VAUGHN, S. F. & BERHOW, M. A. 2005. Glucosinolate hydrolysis products from various plant sources: pH effects, isolation, and purification. *Industrial Crops and Products,* 21**,** 193-202.

VELASCO, P., CARTEA, M. E., GONZÁLEZ, C., VILAR, M. & ORDÁS, A. 2007. Factors Affecting the Glucosinolate Content of Kale (Brassica oleracea acephala Group). *Journal of Agricultural and Food Chemistry,* 55**,** 955-962.

WILLIAMS, D. A. 1982. Extra-binomial variation in logistic linear models. *Applied statistics***,** 144-148.

WILLIAMS, I. 2010. The Major Insect Pests of Oilseed Rape in Europe and Their Management: An Overview. *In:* WILLIAMS, I. H. (ed.) *Biocontrol-Based Integrated Management of Oilseed Rape Pests.* Springer Netherlands.

WILLIAMS, I. H. & FREE, J. B. 1978. The feeding and mating behaviour of pollen beetles ( Meligethes aeneus Fab.) and seed weevils ( Ceutorhynchus assimilis Payk.) on oil-seed rape ( Brassica napus L.). *The Journal of Agricultural Science,* 91**,** 453-459.

WITTSTOCK, U. & BUROW, M. 2010. Glucosinolate Breakdown in Arabidopsis: Mechanism, Regulation and Biological Significance. *The Arabidopsis Book***,** e0134.

# Chapter

# Nomeclature

7

<u>Abbrevations</u>

| | |
|---|---|
| **1MOI3M** | 1-methoxyindol-3-ylmethyl |
| **2PE** | 2-phenylethyl |
| **4MOI3M** | 4-methoxyindol-3-ylmethyl |
| **5MSOP** | 5-methylsulphinylpentyl |
| **ACN** | Acetonitrile |
| **BAR** | Glucobarbarin |
| **C.D** | Control Damaged |
| **CIFs** | Cabbage Identification Factor |
| **Col-0** | Columbia-0 |
| **CSH** | Charged Surface Hybrid |
| **C.UD** | Control Undamaged |
| **ESM** | Epithiospecifier Modifier Protein |
| **ESP** | Epithiospecifier Protein |
| **ES** | Electrospray Ionization |
| **FAO** | Food and Agriculture Organization |
| **FDA** | Food and Drug Administration |
| **GM** | Genetically Modified |
| **GMO** | Genetically Modified Organisms |
| **GRAS** | Generally Recognized as Safe |
| **I3M** | Indol-3-ylmethyl |
| **IAA** | Indoleacetic acid |
| **IS** | Internal Standard |
| **ITCs** | Isothiocyanates |
| **M** | *MINELESS* |
| **MC** | *MINELESS* Control |
| **M.D** | *MINELESS* Damaged |

| | |
|---|---|
| **MDF** | *MINELESS Delia floralis* |
| **MDR** | *MINELESS Delia radicum* |
| **MRM** | Multiple Reaction Monitoring |
| **M.UD** | *MINELESS* Undamaged |
| **NAS** | Gluconasturtiin |
| **OSR odour** | Oilseed Rape Odour |
| **OZTs** | Oxazolidine-thiones |
| **r.h** | Relative Humidity |
| **TQ-S** | Tandem Quadrupole Spectrometer |
| **VOCs** | Volatile Organic Compounds |
| **W** | Wild-type |
| **WC** | Wild-type Control |
| **WDF** | Wild-type *Delia floralis* |
| **WDR** | Wild-type *Delia radicum* |

# Appendix A

*Table A. Weights of biological replicas used in glucosinolate extraction.*

| Treatment | Days of treatment | Biological replicas | Labelling | Weight of the tissue, mg |
|---|---|---|---|---|
| Wild-type control , WC | 4 days | 2 | WC2_4d | 0.1950 |
| | | 3 | WC3_4d | 0.2059 |
| | | 5 | WC5_4d | 0.2099 |
| | | 6 | WC6_4d | 0.2102 |
| | 10 days | 1 | WC1_10d | 0.1960 |
| | | 2 | WC2_10d | 0.1978 |
| | | 5 | WC5_10d | 0.2004 |
| | | 6 | WC6_10d | 0.1956 |
| | 14 days | 2 | WC2_14d | 0.2044 |
| | | 3 | WC3_14d | 0.2025 |
| | | 4 | WC4_14d | 0.2016 |
| | | 5 | WC5_14d | 0.2011 |
| Wild-type *D. radicum*, WDR | 4 days | 2 | WDR2_4d | 0.1980 |
| | | 3 | WDR3_4d | 0.2051 |
| | | 6 | WDR6_4d | 0.2034 |
| | | 7 | WDR7_4d | 0.1618 |
| | 10 days | 2 | WDR2_10d | 0.2005 |
| | | 4 | WDR4_10d | 0.1970 |
| | | 5 | WDR5_10d | 0.2047 |
| | | 9 | WDR9_10d | 0.2039 |
| | 14 days | 3 | WDR3_14d | 0.1997 |
| | | 5 | WDR5_14d | 0.1845 |
| | | 9 | WDR9_14d | 0.2122 |
| | | 10 | WDR10_14d | 0.2047 |

| | | | | |
|---|---|---|---|---|
| **Wild-type *D. floralis*, WDF** | 4 days | 4 | WDF4_4d | 0.1950 |
| | | 8 | WDF8_4d | 0.2005 |
| | | 9 | WDF9_4d | 0.2007 |
| | | 10 | WDF10_4d | 0.2190 |
| | 10 days | 14 | WDF14_10d | 0.1965 |
| | | 19 | WDF19_10d | 0.1930 |
| | | 21 | WDF21_10d | 0.1948 |
| | | 23 | WDF23_10d | 0.2013 |
| | 14 days | 31 | WDF31_14d | 0.2019 |
| | | 35 | WDF35_14d | 0.2005 |
| | | 36 | WDF36_14d | 0.2050 |
| | | 37 | WDF37_14d | 0.2147 |
| *MINELESS* control, MC | 4 days | 2 | MC2_4d | 0.2085 |
| | | 3 | MC3_4d | 0.2019 |
| | | 4 | MC4_4d | 0.2040 |
| | | 5 | MC5_4d | 0.2066 |
| | 10 days | 1 | MC1_10d | 0.1993 |
| | | 2 | MC2_10d | 0.1940 |
| | | 5 | MC5_10d | 0.2057 |
| | | 6 | MC6_10d | 0.2058 |
| | 14 days | 2 | MC2_14d | 0.2030 |
| | | 3 | MC3_14d | 0.2049 |
| | | 4 | MC4_14d | 0.2059 |
| | | 5 | MC5_14d | 0.2077 |
| *MINELESS* *D. radicum*, MDR | 4 days | 2 | MDR2_4d | 0.1957 |
| | | 3 | MDR3_4d | 0.2176 |
| | | 4 | MDR4_4d | 0.2065 |
| | | 8 | MDR8_4d | 0.2033 |
| | 10 days | 19 | MDR19_10d | 0.1982 |
| | | 25 | MDR25_10d | 0.1940 |
| | | 26 | MDR26_10d | 0.1948 |

| | | 27 | MDR27_10d | 0.2034 |
|---|---|---|---|---|
| | 14 days | 28 | MDR28_14d | 0.2036 |
| | | 32 | MDR32_14d | 0.2066 |
| | | 33 | MDR33_14d | 0.2060 |
| | | 36 | MDR36_14d | 0.2056 |
| *MINELESS D. floralis*, **MDF** | 4 days | 26 | MDF26_4d | 0.2005 |
| | | 30 | MDF30_4d | 0.2034 |
| | | 32 | MDF32_4d | 0.2003 |
| | | 33 | MDF33_4d | 0.1988 |
| | 10 days | 13 | MDF13_10d | 0.1949 |
| | | 19 | MDF19_10d | 0.1946 |
| | | 22 | MDF22_10d | 0.2097 |
| | | 24 | MDF24_10d | 0.2033 |
| | 14 days | 1 | MDF1_14d | 0.1933 |
| | | 8 | MDF8_14d | 0.2129 |
| | | 12 | MDF12_14d | 0.2095 |
| | | 25 | MDF25_14d | 0.1975 |

# Appendix B

*Table B 1: Free choice experiment 1. Consumption of cotyledon (cm$^2$) by M. brassicae larvae.*

| Consumed area of wild-type cotyledons | Consumed area of MINELESS cotyledons |
|---|---|
| 0.679 | 0.359 |
| 1.14 | 0.487 |
| 0.876 | 0.344 |
| 0.685 | 0.372 |
| 0.6 | 0.186 |
| 0.902 | 0.151 |
| 0.639 | 0.214 |
| 0.654 | 0.321 |
| 0.426 | 0.174 |
| 0.788 | 0.243 |
| 0.509 | 0.115 |
| 0.443 | 0.232 |
| 0.426 | 0.266 |
| 0.379 | 0.284 |
| 0.431 | 0.208 |

*Table B 2: Free choice experiment 2. Consumption of cotyledon (cm2) by M. brassicae larvae.*

| Consumed area of wild-type cotyledons | Consumed area of MINELESS cotyledons |
|---|---|
| 1.009 | 0.577 |
| 1.015 | 0.259 |
| 0.543 | 0.284 |
| 0.247 | 0.145 |
| 0.27 | 0.559 |
| 0.348 | 0.16 |
| 0.325 | 0.362 |
| 0.28 | 0.376 |
| 0.266 | 0.183 |
| 0.358 | 0.439 |
| 0.447 | 0.277 |
| 0.376 | 0.133 |
| 0.425 | 0.194 |
| 0.338 | 0.157 |
| 0.341 | 0.182 |

# Appendix C

*Table C 1: Pollen beetles feeding damage to B. napus buds in Feeding experiment I.*

| Rep | Number of beetles retrieved | | | Sex of the beetles | | Number of buds | Number of feeding damage on buds | Number of flowers | Number of damage in flowers | Total number of buds + flowers | Total number of damage in buds + flowers | Total number of damage/total number of buds + flowers x 100% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Number* | *alive* | *dead* | *Males (dead)* | *Females (dead)* | | | | | | | |
| 1 | 2 | 2 | 0 | 2 | 0 | 45 | 0 | 4 | 0 | 49 | 0 | - |
| 1 | 2 | 2 | 0 | 1 | 1 | 32 | 0 | 4 | 1 | 36 | 1 | 2.78 |
| 1 | 2 | 2 | 0 | 1 | 1 | 56 | 0 | 9 | 1 | 65 | 1 | 1.54 |
| 1 | 2 | 2 | 0 | 2 | 0 | 49 | 0 | 6 | 0 | 55 | 0 | - |
| 2 | 2 | 2 | 0 | 1 | 1 | 47 | 0 | 5 | 1 | 52 | 1 | 1.92 |
| 2 | 2 | 2 | 0 | 1 | 1 | 42 | 0 | 5 | 1 | 47 | 1 | 2.13 |
| 2 | 2 | 2 | 0 | missing | missing | 60 | 0 | 5 | 0 | 65 | 0 | - |
| 2 | 2 | 2 | 0 | 0 | 2 | 45 | 0 | 6 | 1 | 51 | 1 | 1.96 |
| 3 | 2 | 2 | 0 | 1 | 1 | 44 | 0 | 5 | 0 | 49 | 0 | - |
| 3 | 2 | 2 | 0 | 2 | 0 | 26 | 0 | 1 | 0 | 27 | 0 | - |
| 3 | 2 | 2 | 0 | 1 | 1 | 50 | 0 | 10 | 0 | 60 | 0 | - |
| 3 | 2 | 2 | 0 | 1 | 1 | 48 | 0 | 11 | 1 | 59 | 1 | 1.69 |
| 4 | 2 | 2 | 0 | 0 | 2 | 46 | 0 | 4 | 0 | 50 | 0 | - |
| 4 | 2 | 2 | 0 | 1 | 1 | 45 | 0 | 5 | 1 | 50 | 1 | 2.00 |
| 4 | 0 | 0 | 0 | 0 | 0 | 40 | 0 | 8 | 0 | 48 | 0 | - |
| 4 | 2 | 2 | 0 | 1 | 1 | 63 | 1 | 8 | 1 | 71 | 2 | 2.82 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 | 2 | 2 | 0 | 0 | 2 | 54 | 0 | 4 | 1 | 58 | 1 | 1.72 |
| 5 | 2 | 1 | 1 | 2 | 0 | 44 | 0 | 5 | 0 | 49 | 0 | - |
| 5 | 1 | 1 | 0 | 1 | 0 | 52 | 0 | 8 | 0 | 60 | 0 | - |
| 5 | 2 | 2 | 0 | 0 | 2 | 51 | 0 | 8 | 0 | 59 | 0 | - |
| 6 | 2 | 2 | 0 | 1 | 1 | 45 | 0 | 5 | 3 | 50 | 3 | 6.00 |
| 6 | 2 | 2 | 0 | 1 | 0 | 44 | 0 | 1 | 0 | 45 | 0 | - |
| 6 | 2 | 2 | 0 | 2 | 0 | 60 | 0 | 11 | 0 | 71 | 0 | - |
| 6 | 2 | 2 | 0 | 2 | 0 | 45 | 0 | 6 | 0 | 51 | 0 | - |
| 7 | 2 | 2 | 0 | 1 | 1 | 50 | 1 | 5 | 0 | 55 | 1 | 1.82 |
| 7 | 2 | 2 | 0 | 1 | 1 | 53 | 3 | 4 | 0 | 57 | 3 | 5.26 |
| 7 | 2 | 2 | 0 | 1 | 1 | 62 | 2 | 13 | 0 | 75 | 2 | 2.67 |
| 7 | 0 | 0 | 0 | 0 | 0 | 50 | 2 | 10 | 0 | 60 | 2 | 3.33 |
| 8 | 2 | 2 | 0 | 1 | 1 | 54 | 0 | 6 | 1 | 60 | 1 | 1.67 |
| 8 | 2 | 2 | 0 | 1 | 1 | 26 | 0 | 0 | 0 | 26 | 0 | - |
| 8 | 2 | 2 | 0 | 1 | 1 | 57 | 0 | 11 | 0 | 68 | 0 | - |
| 8 | 2 | 2 | 0 | 2 | 0 | 58 | 0 | 6 | 0 | 64 | 0 | - |
| 9 | 2 | 2 | 0 | 1 | 1 | 40 | 0 | 7 | 2 | 47 | 2 | 4.26 |
| 9 | 2 | 2 | 0 | 1 | 1 | 54 | 1 | 4 | 0 | 58 | 1 | 1.72 |
| 9 | 2 | 2 | 0 | 1 | 1 | 42 | 0 | 8 | 1 | 50 | 1 | 2.00 |
| 9 | 1 | 1 | 0 | 2 | 0 | 52 | 0 | 5 | 0 | 57 | 0 | - |
| 10 | 2 | 2 | 0 | 1 | 1 | 35 | 0 | 7 | 1 | 42 | 1 | 2.38 |
| 10 | 2 | 2 | 0 | 1 | 1 | 46 | 0 | 3 | 0 | 49 | 0 | - |
| 10 | 2 | 2 | 0 | 1 | 1 | 55 | 0 | 10 | 2 | 65 | 2 | 3.08 |
| 10 | 2 | 2 | 0 | damaged | 1 | 51 | 0 | 12 | 1 | 63 | 1 | 1.59 |
| 11 | 1 | 1 | 0 | 0 | 1 | 34 | 1 | 4 | 1 | 38 | 2 | 5.26 |
| 11 | 2 | 1 | 1 | damaged | 1 | 32 | 0 | 4 | 0 | 36 | 0 | - |
| 11 | 2 | 2 | 0 | 1 | 1 | 60 | 0 | 13 | 0 | 73 | 0 | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | 2 | 1 | 1 | 1 | 1 | 35 | 0 | 6 | 0 | 41 | 0 | - |
| 12 | 2 | 2 | 0 | 2 | 0 | 43 | 0 | 7 | 0 | 50 | 0 | - |
| 12 | 2 | 2 | 0 | 2 | 0 | 45 | 0 | 7 | 0 | 52 | 0 | - |
| 12 | 2 | 2 | 0 | 1 | 1 | 55 | 0 | 9 | 0 | 64 | 0 | - |
| 12 | 2 | 1 | 1 | previously damaged insect | 1 | 54 | 0 | 13 | 2 | 67 | 2 | 2.99 |
| 13 | 2 | 2 | 0 | 1 | 1 | 48 | 0 | 8 | 0 | 56 | 0 | - |
| 13 | 2 | 2 | 0 | 1 | 1 | 28 | 0 | 3 | 0 | 31 | 0 | - |
| 13 | 2 | 2 | 0 | 1 | 1 | 70 | 0 | 14 | 0 | 84 | 0 | - |
| 13 | 2 | 2 | 0 | 0 | 2 | 65 | 0 | 6 | 0 | 71 | 0 | - |
| 14 | 2 | 2 | 0 | 0 | 2 | 45 | 0 | 7 | 0 | 52 | 0 | - |
| 14 | 2 | 2 | 0 | 2 | 0 | 39 | 1 | 0 | 0 | 39 | 1 | 2.56 |
| 14 | 2 | 2 | 0 | 2 | 0 | 22 | 0 | 8 | 2 | 30 | 2 | 6.67 |
| 14 | 2 | 1 | 1 | 1 | 1 | 65 | 0 | 7 | 0 | 72 | 0 | - |
| 15 | 2 | 2 | 0 | 0 | 2 | 36 | 0 | 4 | 0 | 40 | 0 | - |
| 15 | 2 | 2 | 0 | 0 | 2 | 33 | 0 | 5 | 0 | 38 | 0 | - |
| 15 | 2 | 2 | 0 | missing | 1 | 55 | 0 | 5 | 0 | 60 | 0 | - |
| 15 | 2 | 2 | 0 | 1 | 1 | 53 | 0 | 8 | 0 | 61 | 0 | - |

*Table C 2: Pollen beetles feeding damage to B. napus buds in Feeding experiment II.*

| Plant treatment | Total number of buds | Number of damage |
|:---:|:---:|:---:|
| W1 | 41 | 1 |
| M1 | 48 | 0 |
| W2 | 33 | 1 |
| M2 | 40 | 6 |
| W3 | 42 | 4 |
| M3 | 54 | 7 |
| W4 | 37 | 5 |
| M4 | 44 | 5 |
| W5 | 65 | 0 |
| M5 | 48 | 4 |
| W6 | 50 | 1 |
| M6 | 50 | 5 |
| W7 | 37 | 9 |
| M7 | 52 | 0 |
| W8 | 44 | 7 |
| M8 | 56 | 0 |
| W9 | 41 | 1 |
| M9 | 47 | 0 |
| W10 | 34 | 6 |
| M10 | 70 | 0 |
| W11 | 49 | 6 |
| M11 | 45 | 0 |
| W12 | 37 | 6 |
| M12 | 46 | 2 |
| W13 | 37 | 2 |
| M13 | 49 | 9 |
| W14 | 37 | 0 |
| M14 | 53 | 2 |
| W15 | 75 | 2 |
| M15 | 52 | 0 |
| W16 | 48 | 0 |
| M16 | 50 | 7 |
| W17 | 36 | 5 |
| M17 | 19 | 1 |
| W18 | 45 | 3 |

| | | |
|---|---|---|
| M18 | 50 | 1 |
| W19 | 41 | 4 |
| M19 | 69 | 0 |
| W20 | 40 | 6 |
| M20 | 41 | 4 |
| W21 | 36 | 0 |
| M21 | 37 | 5 |
| W22 | 38 | 0 |
| M22 | 43 | 4 |
| W23 | 41 | 4 |
| M23 | 55 | 2 |
| W24 | 42 | 0 |
| M24 | 33 | 2 |
| W25 | 39 | 0 |
| M25 | 47 | 1 |
| W26 | 38 | 6 |
| M26 | 30 | 1 |
| W27 | 44 | 0 |
| M27 | 53 | 2 |
| W28 | 36 | 6 |
| M28 | 51 | 6 |
| W29 | 44 | 2 |
| M29 | 53 | 2 |
| W30 | 39 | 11 |
| M30 | 48 | 4 |
| W31 | 34 | 9 |
| M31 | 54 | 5 |
| W32 | 57 | 0 |
| M32 | 47 | 9 |

*Table C 3: Choice of pollen beetles in Y-tube olfactometer experiment.*

| Beetle | Choice | | | Oilseed rape odour (on the right_R or on the left_L) |
|---|---|---|---|---|
| | Left | Right | Non-responder | |
| 1 | - | 1 | - | R |
| 2 | - | 1 | - | R |
| 3 | - | - | 1 | R |
| 4 | 1 | - | - | L |
| 5 | 1 | - | - | L |
| 6 | 1 | - | - | L |
| 7 | 1 | - | - | R |
| 8 | 1 | - | - | R |
| 9 | 1 | - | - | R |
| 10 | 1 | - | - | L |
| 11 | - | - | 1 | L |
| 12 | - | 1 | - | L |
| 13 | - | 1 | - | R |
| 14 | - | - | 1 | R |
| 15 | - | 1 | - | R |
| 16 | 1 | - | - | L |
| 17 | 1 | - | - | L |
| 18 | 1 | - | - | L |