Effects of nitrogen fertilization on insect pests, their parasitoids, plant diseases and volatile organic compounds in *Brassica napus*

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**A R T I C L E   I N F O**

Article history:
Received 25 May 2012
Received in revised form 31 August 2012
Accepted 3 September 2012

Keywords:
N fertilization
VOC emission
*Meligethes aeneus*
*Ceutorhynchus obstrictus*
*Alternaria brassicae*
Winter oilseed rape

**A B S T R A C T**

Nitrogen (N) availability is a key factor influencing the yield of *Brassica napus* L. Thus, mineral fertilization is widely used to improve the quality and quantity of seeds. In this study, we conducted field experiments to determine the impact of nitrogen fertilization on *B. napus* pests, their parasitoids and plant diseases. The results showed that N treatment had an impact on the abundance of pollen beetles (*Meligethes aeneus* Fab.) and cabbage seed weevils (*Ceutorhynchus obstrictus* Marsh.) as well as dark spot disease (*Alternaria brassicae* (Berk.) Sacc.). Since pest abundance was not correlated with the flower and silique numbers, the feeding and oviposition sites, plant smell bouquets were analysed to determine potentially attractive or repellent volatile organic compounds. We detected 19 different compounds among which acetic acid and several lipoxygenase pathway products were emitted at higher levels from N-treated plants. Emission of a few other terpenoid compounds was correlated with the pest abundance in field conditions. Abundance of parasitoids of both pests was related to the host availability rather than to the fertilization treatment. Therefore, we suggest that plant chemical cues play a minor role in localization of hosts in close proximity to parasitoid. Dark spot disease levels decreased with increasing N availability, possibly reflecting enhanced emissions of acetic acid, a known antifungal volatile. This study demonstrates the effects of N fertilization on bud and flower volatile bouquets, which might play a role in *B. napus* insect pest host selection and in resistance to fungal plant diseases. Further studies are necessary to investigate the behavioural responses of insects to the changed volatile bouquets.

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1. Introduction

Oilseed rape (*Brassica napus* L.) is a widely cultivated field crop in temperate climates (Blake et al., 2010) where its growth and development is most commonly limited by nitrogen (N) availability, especially at the onset of vegetative growth (e.g. Holmes, 1980; Rathke et al., 2006; Sieling and Kage, 2010). Conventionally, mineral fertilizers are applied to improve the yield and biochemical characteristics of this important agricultural crop. Fertilization with mineral N has been shown to increase plant size, height and characteristics of this important agricultural crop. Fertilization with mineral fertilizers are applied to improve the yield and biochemical development is most commonly limited by nitrogen (N) availability, possibly relocalization of hosts in close proximity to parasitoid. Dark spot disease levels decreased with increasing N availability, possibly reflecting enhanced emissions of acetic acid, a known antifungal volatile. This study demonstrates the effects of N fertilization on bud and flower volatile bouquets, which might play a role in *B. napus* insect pest host selection and in resistance to fungal plant diseases. Further studies are necessary to investigate the behavioural responses of insects to the changed volatile bouquets.

Among *B. napus* pests, the pollen beetle (*Meligethes aeneus* Fab.), Coleoptera: Nitidulidae) and the cabbage seed weevil (*Ceutorhynchus assimilis* Payk.), Coleoptera: Curculionidae) are the most widespread and persistent pests in the UK, Central, North and North Eastern Europe (Alford et al., 2003; Veromann et al., 2006b; Williams, 2010).
Pollen beetle adults feed on pollen, destroy flower buds and lay eggs into the buds where their larvae feed on pollen. Seed weevils oviposit in developing siliques and their larvae consume seeds. The management of these B. napus pests is generally conducted by chemical pesticides, although their abundance could be efficiently controlled by their natural enemies, parasitoids (Büchi, 2002; Veromann et al., 2011; Walters et al., 2003). Parasitoids are insects, mostly from the order Hymenoptera, laying eggs on or inside the insects of other species. Their larvae develop and feed on the host, eventually killing it (Godfray, 1994). In Europe, the larvae of M. aeneus are parasitized by at least nine species of endoparasitoids. Among these, Phadris interstitialis (Thomson), Phadris morionellus (Holmgren), Tersilochus heterocerus (Thomson) and Diospilus capito (Nees) (Hymenoptera, Ichneumonoidea) are the most abundant and widespread (Nilsson, 2003). The most common parasitoids of C. obstrictus are larval ectoparasitoids belonging to the superfamily Chalcidoidea: Trichomalus perfectus (Walker), Mesopolobus morys (Walker) and Stenomalina gracilis (Walker) (Williams, 2003).

Among B. napus diseases, the most devastating are blackleg (Leptosphaeria maculans (Desm.) Ces.) and stem rot (Sclerotinia sclerotiorum (Lib.) de Bary). Additionally, the dark spot disease (Alternaria brassicae (Berk.) Sacc.) is a common fungal pathogen, especially on winter varieties (Giamoustaris and Mithen, 1997; Köhl et al., 2010). Disease control in conventional cropping systems is mainly conducted by preventive fungicide applications throughout the season, often without considering the presence or abundance of pathogens. So far, it has been assumed that N usage is unlikely to have any direct effect on the abundance of the B. napus insect pests (Walters et al., 2003) and only a limited number of studies has focussed on the effects of N fertilization on parasitism level of some lepidopteran’s and aphids’ pests (Jansson, 2003; Kalule and Wright, 2002; Stanley et al., 2011). For instance, the amount of nitrogen supplied to Brassica oleracea var. capitata has been shown to increase the parasitism level of Plutella xylostella (Lepidoptera: Plutellidae) by Diadegma insulare (Cresson) (Hymenoptera: Ichneumonidae) (Jansson et al., 1991). However, fertilization changes plant architecture and inner microclimate of the crop, which might influence the selection efficiency of both the insect pests and their hymenopterous parasitoids (Walters et al., 2003). Additionally, plant architecture can have an impact on the distribution and accessibility of host larvae within the stand and therefore may affect the host location success of the parasitoids (Ulker and Fisher, 2006). There is some evidence of either negative or positive effects of N on stem rot disease and blackleg (Aubertot et al., 2003; Rathke et al., 2006; Söchting and Verreet, 2004), although the impact of fertilization on dark spot disease was non-significant in previous experiments (Söchting and Verreet, 2004).

Chemical communication between herbivores and their host plants depends on the plant and herbivore species and is generally based on multiple compounds (Blight et al., 1997). Cruciferous plants, such as B. napus, emit a complex mixture of biogenic volatile organic compounds (VOCs). Among them, isothiocyanates from foliage and floral compounds are known to be important cues in host selection by cruciferous pests and their parasitoids, aiding both finding and recognition of the host plant (Alford et al., 2003; Bartlet et al., 1993; Schiestl, 2010). Nitrogen fertilization could significantly affect the composition and levels of plant VOCs (Chen et al., 2010) and therefore also affect their attractiveness to pests. Our preliminary results on the effect of N show that there might be a correlation between the nutrient application and the abundance of pests, diseases and even parasitoids (Veromann et al., in press). Thus, it is necessary to conduct additional experiments to determine the factors involved in the pest and disease abundance, which could be further used to control the damage to the crop without the use of pesticides.

In this study we hypothesized that N fertilization affects the abundance of pests and parasitoids, as well as the spread of plant diseases, and that these potential modifications are associated with changes in the VOC bouquets of the plants. To test this hypothesis, we determined the VOC emissions of B. napus plants under laboratory conditions and compared these with the field data on abundance of insect pests, their parasitoids and plant diseases under different N treatments. The results of this study provide evidence that the composition of the VOCs emitted from B. napus plants is linked to the N fertilization effects on insect damage and dark spot disease.

2. Material and methods

2.1. Field experiment setup and yield estimation

The field studies were conducted in an experimental field of Jõgeva Plant Breeding Institute, Estonia (58°46’01”N, 26°24’27”E; elevation 74 m) in 2008 and 2009. The study site was ploughed and kept as a fallow for one growing season before starting this experiment. On the 23 April of each year, seven different N-fertilizer levels of 0, 60, 80, 100, 120, 140 and 160 kg of N (applied as NH4NO3 (Akron Group, Novgorod, Russia)) per hectare were applied on three randomized replicate plots of 10 m². Thus, the study design consisted of seven nitrogen treatments (hereinafter indicated as N0, N60, N80, N100, N120, N140, N160) for a total of 21 plots. Before sowing the seed in 2007 and in 2008 (for the 2008 and 2009 harvest, respectively), all the plots were fertilized with Kemira Power (Yara International ASA, Norway) 5—10—25 Se B complex fertilizer (300 kg ha⁻¹). In both years, a winter oilseed rape variety B. napus cv. Silva seeds were drilled on the 15 August in 2007 (for 2008 harvest) and on the 15 August in 2008 (for 2009 harvest) with seeding rate of 6 kg ha⁻¹. No insecticides or fungicides were applied.

The crop was harvested on the 7 September in 2008 and on the 11 August in 2009. The seed mass was measured for every plot by weighing the seeds at a moisture content of 7.5%. Thereafter, the yield per each plot was calculated as kg ha⁻¹. In addition, the number of siliques was counted. Siliquing counting was conducted at the pods ripening stage (plant growth stage BBCH 80—81 according to Lancashire et al., 1991) on three plants from each plot.

2.2. Quantification of pests, parasitoids and plant diseases

For estimation of egg laying activity and larval parasitization levels of M. aeneus, larvae were collected from B. napus flowers at stage BBCH 67–68 (at the end of full flowering) from five randomly chosen plants in each plot. Larvae were counted and second instar larvae were dissected in laboratory to determine their parasitization. Second instar is the last M. aeneus larval stage before dropping to the ground to pupate in the soil. Thus, by this time their parasitoids must have had found a suitable host to parasitize.

The establishment of damage and parasitization of C. obstrictus was assessed at stage BBCH 80—81 (beginning of pods ripening). Five siliques from the main raceme and five siliques from the third side branch were collected from five randomly chosen plants per plot and incubated in emergence traps in the laboratory as described in detail in Veromann et al. (2011). Four weeks later, emerged adult parasitoids or weevils’ larvae were counted and identified. The exit-holes of larvae and parasitoids in all siliques were counted. Thereafter, all siliques were dissected and remains of any non-exited weevil larvae or the parasitoid pupae were noted. Finally, the mean number of emerged parasitoids, damaged siliques
by *C. obstrictus* and the percentage of parasitized weevils were calculated per plant.

Visual disease assessments were made at the mature pod stage (BBCH 80–85) when five plants were randomly chosen from every plot. The abundance of *A. brassicae* lesions on leaves, stems and siliques was visually assessed on a qualitative scale from 0 to 6 (0 = no disease; 1 = 1–5%; 2 = 5–10%; 3 = 10–20%; 4 = 20–30%; 5 = 30–50%; 6 = more than 50% of surface area covered with lesions), based on the assessment key illustrated in Conn et al. (1990).

### 2.3. Volatile organic compound measurements

The VOC measurements were carried out only in May 2010 using the plants from the same *B. napus* variety grown in similar experimental setup as described above. Since the plants were treated similarly to the ones in field studies in 2008 and 2009, we used the data from VOC analyses to determine potential volatile cues in plant–insect relationships and disease abundance that were detected in field studies during earlier years. To detect and compare VOCs from plants with different fertilization levels, five well overwintered plants from treatments N0, N80, N100 and N160 were taken from the field on the 4th May (stem elongation stage BBCH 33–35). The plants were planted in 5 L pots with the soil from the field and placed in a growth room where they were protected from biogenic stressors and watered every second day. The plants were grown under metal halide lamps (HPI-T Plus 400 W, Philips). At plant level, the light intensity was 300 µmol m⁻² s⁻¹ for 12 h light period and day/night temperatures of 24/18 °C.

The first set of VOC measurements was made on the 17th May, when the plants were in bud stage (BBCH 51–53) and the second set of measurements on the 20th May when the plants were flowering (BBCH 63–65). For both sets of measurements, three plants from every treatment were chosen with similar number of buds or flowers. The measurements were carried out in the morning, between 9.00 a.m. and 11.00 a.m. The top of main raceme of one plant from every treatment at a time was placed into a multi-chamber cuvette system described in Toome et al. (2010) and the volatiles were sampled by concentrating the air from every chamber into a multibed stainless steel cartridge. The adsorption was carried out at a flow rate of 200 ml min⁻¹ for 20 min with a constant flow air sample pump (SKC Inc., Houston, TX, USA). Thereafter the same procedure was repeated with the other two sets of plants, each containing one plant from every N0, N80, N100 and N160 treatment. Additional samples were taken from the inlet air stream prior to the chambers to determine the background value of the VOCs.

The adsorbed cartridges were analysed with a combined Shimadzu TD20 automated cartridge desorber and Shimadzu 2010 plus GC MS instrument (Shimadzu Corporation, Kyoto, Japan) using the method described in detail previously (Copolovici et al., 2009, 2011; Toome et al., 2010). The compounds were identified by comparing their mass spectra with a NIST library (National Institute of Standards and Technology) and with authentic standards. VOC emission rates were expressed per bud or flower projected area enclosed in the chamber.

### 2.4. Data analysis

The differences among the means of the number of siliques, yields, damaged siliques, parasitoids of *C. obstrictus* and larvae of *M. aeneus* between the treatments were tested by Wald Statistics using Type III empirical standard error analysis with the Poisson distribution and the logit-link function. Differences among the means between years and treatments were tested by the GENMOD procedure Differences of Least Squares Means test. The average numbers of parasitized larvae of *M. aeneus* were compared by the same analysis, but with Binomial distribution and logit-link function with second instar larvae as the response variable. The scale parameter was estimated by Pearson Chi-Square divided by the degrees of freedom to account for the model overdispersion. Spearman correlation coefficient *r* was calculated to test for the correlations between the number of *M. aeneus* larvae and their parasitism level, and between damaged siliques and parasitism level of *C. obstrictus* larvae. Additionally, correlations between the abundance of both pests and the volatile emission rates measured in the laboratory conditions were calculated. The impact of treatment on the mean disease scores was determined with a nonparametric Kruskal–Wallis Test. In addition, Spearman correlation coefficients were calculated to describe the correlation between the fertilizer application level and disease scores. These statistical analyses were carried out using GLM and GENMOD procedures in SAS 8.02 (SAS Institute, Inc., Cary, NC, USA).

The impact of nitrogen fertilization on the emission of various volatiles was visualized by fitting data in a non-linear regression model in the exponential form, using OriginPro 8 (OriginLab Corporation, Northampton, MA, USA). The smell bouquets of volatiles (based on the emission rates) released by main racemes at bud and flowering stage were explored by principal component analysis (PCA) (Wold et al., 1987). After mean-centering, square-root data transformation was used. The resulting loading and score plots were derived for volatile bouquets at bud and flower stages for different N treatments. The difference between the emission bouquets from plants at flowering and bud stage was tested by Monte-Carlo permutation test using redundancy data analysis (RDA). The multivariate analyses were conducted by Canoco 4.5 software (ter Braak and Smilauer, Biomimics Plant Research International, The Netherlands). All statistical tests were considered significant at *P* < 0.05.

### 3. Results

#### 3.1. Impact of fertilization on the number of siliques

Fertilization significantly increased the number of siliques and yield (Table 1). Plants in control plots with no external N-supply had the smallest number of siliques and the smallest yield (*χ² = 53.49, df = 6, P < 0.0001*). The highest number of siliques was found from treatments N100 to N160 with no significant differences between these treatments (Table 1). The plants in N160 plots had nearly three times more siliques than the plants growing in N0 plots (*χ² = 46.76, df = 1, P < 0.0001*). Overall, the ranking of treatments according to the number of siliques and yield corresponded to the fertilization level, although not always. Year effect was significant in most cases for yield and for N120 for number of siliques, but the qualitative trend of increased number of siliques and yield was the same in both years (Table 1).

#### 3.2. Insect pests and their parasitoids

Overall, the oviposition activity of *M. aeneus* as well as the parasitism level of their larvae was relatively low. The mean number of *M. aeneus* larvae (*χ² = 63.80, df = 6, P < 0.0001*) and parasitized larvae per plant (*χ² = 78.45, df = 6, P < 0.0001*) differed significantly among N fertilization treatments (Fig. 1a, b, c). Although the number of larvae was significantly greater in 2009 compared to 2008 (*χ² = 32.18, df = 1, P < 0.0001*), the general distribution of larvae between treatments had a similar pattern (Fig. 1a, b). In 2008, the number of larvae per plant was extremely low and only the treatments N80 (*χ² = 3.75, df = 1, P = 0.05*) and N160 (*χ² = 4.54, df = 1, P = 0.03*) had significantly greater number
of larvae per plant than N0 (Fig. 1a). In 2009, the number of larvae was greater and the treatments N60, N80 and N160 clearly differed from the remaining treatments (P < 0.01; Fig. 1b). Similarly to the larvae, the parasitism percentage differed significantly between the years (χ² = 38.32, df = 6, P < 0.0001; Fig. 1a, b). In 2008, the maximum percentage reached to 9.76 in N160 and differed significantly from N80 (χ² = 4.79, df = 1, P < 0.05; Fig. 1a), whereas in 2009, parasitism percentage reached 47.62 in N0, differing significantly from all other treatments, except for the treatment N80 (χ² = 2.74, df = 1, P > 0.05; Fig. 1b). When analysing the data from two years together, the greatest numbers of M. aeneus were found in plots N60, N80 and N160 (P < 0.05; Fig. 1c). Although the smallest number of larvae was found in the flowers of the plants from treatment N0, no significant difference between larval number was detected between the treatments N0 and N140 (χ² = 2.00, df = 1, P > 0.05). The mean parasitism rate averaged across years studied was the greatest in the least M. aeneus infested plants in plots N0, where 26% of larvae were parasitized. A statistically significant difference in parasitism level was found only between treatments N0 and N100 (χ² = 4.71, df = 1, P < 0.05; Fig. 1c). A significant positive correlation was detected between the number of pollen beetle larvae and the number of parasitized larvae (r = 0.24; P < 0.01).

The mean percentage of siliques damaged by C. obstrictus varied from 8.3 to 14.3%. Fertilization with nitrogen affected significantly the number of damaged siliques per plant averaged across years (χ² = 12.71, df = 6, P < 0.05; Fig. 1f). Averaged across years, the mean number of C. obstrictus damaged siliques was higher in the treatment N80 than in the treatments N100 (χ² = 7.64, df = 1, P < 0.01) and N140 (χ² = 7.64, df = 1, P < 0.01; Fig. 1f). In 2008, the number of siliques damaged by C. obstrictus was extremely low and due to high variability there were no significant differences between the treatments (Fig. 1d). In 2009, significantly more damaged siliques was found than in 2008 (χ² = 9.51, df = 1, P < 0.01; Fig. 1e). The least damaged plants were in plots N100 and N140 and they differed significantly from the treatment N80 (accordingly: χ² = 6.34, df = 1, P < 0.05 and χ² = 3.80, df = 1, P > 0.05). The mean parasitism percentage of the C. obstrictus larvae was extremely high, varying from 76% to 94% with an average of 86.4 ± 2.8%. In 2008–2009, the greatest number of parasitoids was found in the treatments N60 (χ² = 4.31, df = 1, P < 0.05), N80 (χ² = 5.54, df = 1, P < 0.05) and N120 (χ² = 3.93, df = 1, P < 0.05), where significantly more parasitoids emerged from siliques than in plot N140 (Fig. 1f). A strong positive correlation between host availability (damaged siliques) and parasitism level was found across all nitrogen treatments when pooled (r = 0.94, P < 0.0001). In 2008, the number of emerged parasitoids was equal with the number of damaged siliques almost in all treatments. Thus, the parasitism level was 100% and there were no differences between treatments (Fig. 1d). In the next study year, the number of emerged parasitoids was somewhat smaller and the parasitism level varied between 63.9 and 95.5% (Fig. 1e). There were no differences between the two study years (χ² = 2.21, df = 1, P = 0.14).

3.3. Diseases

The only B. napus disease found during this two-year study was dark spot disease, a fungal infection caused by A. brassicaceae. This disease occurred on leaves, stems and siliques. The average disease score was rather low (Fig. 1h), yet it significantly depended on nitrogen fertilization (χ² = 64.90, df = 6, P < 0.0001). During the first study year, the highest disease scores were detected in plots N0 and N60 and the plants treated with greater nitrogen levels had significantly less disease lesions (P < 0.05; Fig. 1g). In the following year, there was no difference between the control plants and plants with highest nitrogen treatment. However, plants in N80 plots were the least infected (compared to N0: χ² = 64.90, df = 1, P < 0.05; N60: χ² = 4.48, df = 1, P < 0.05; N100: χ² = 4.48, df = 1, P < 0.05; N120: χ² = 6.29, df = 1, P < 0.05; N140: χ² = 13.12, df = 1, P < 0.01; N160: χ² = 28.17, df = 1, P < 0.01).

3.4. Emissions of volatile organic compounds in relation to N treatment

Altogether 19 different VOCs were detected from the oilseed rape racemes during this study (Table 2). The highest emission rates were detected for acetic acid, 3-carene, α-pinene and benzaldehyde (Table 2). These compounds dominated the emissions from plants in both bud and flower stages. For individual compounds, the treatment effect was moderate or small in most cases (Table 2). Nevertheless, emissions of several compounds increased significantly with increasing N availability, in particular LOX, α-thujene and acetic acid emission rates (Fig. 2).

The bouquets were not clearly separated among different N treatments as the bouquets from N-treated plants clustered together with controls on PCA (Fig. 3). Plants in flowering stage treated with higher N levels clustered separately from controls, reflecting their greater (3Z)-hexenol, α-thujene and acetic acid emission rates (Fig. 3).

In average, several statistically significant correlations between the bud stage volatiles and abundance of larvae of M. aeneus and between the flower stage VOC and the damaged siliques by C. obstrictus were found (Table 3). In particular, there were significant negative correlations between the abundance of M. aeneus larvae and β-pinene, linalool, (E,E)-α-farnesene, benzaldehyde and methylbenzoate. Larval abundance was positively correlated with (3Z)-hexenyl acetate, camphene, 3-carene, limonene, indole and acetic acid emission rates. Statistically significant but weak positive
correlations were found between α-pinene, camphene, β-pinene emissions and the abundance of damaged siliques by *C. obstrictus*. There were some differences among years 2008 and 2009 in the correlations of the bud stage volatiles vs. the abundance of larvae of *M. aeneus*. In the first year, significant correlations of larval abundance vs. β-pinene, linalool, (E,E)-α-farnesene, benzaldehyde and methylbenzoate were observed. In 2009, α-pinene not correlated with larval abundance (Table 3). In that year, statistically significant
but weak positive correlations were found between the abundance of damaged siliques by C. obstrictus and the emission of α-pinene, camphene and β-pinene.

3.5. Differences in emission signatures between bud and flower stages

Important differences in VOC emissions from racemes were detected between the bud and flower stages (Table 2 for full statistical analysis). For instance, α-thujene emission was detected only while the plants were flowering and not in the bud stage. Additionally, the mean emissions of several other monoterpenes, i.e., α-pinene (F2,2 = 17.64, P < 0.05), β-pinene (F2,2 = 18.37, P < 0.05), 3-carene (F2,2 = 2.14, P < 0.05), were detected at considerably higher rates during the bud stage compared to the flower stage. The mean emission rate of acetic acid was higher in the flowering stage than in the bud stage. In contrast, the emission rates of camphene and methyl salicylate were higher in the bud stage compared to the flowering stage (F1,2 = 946.70, P < 0.05 and F2,2 = 8.26, P < 0.05, respectively, Table 2).

PCA analysis demonstrated that VOC bouquets of B. napus plants at bud and flowering stage are clearly separated (Fig. 3) and statistically different (Monte Carlo permutation test, P < 0.05). There was a large variation within the bouquet of volatiles released at these two different stages. The distribution of plants on score plot was mainly influenced by the emission rate of (3Z)-hexenyl acetate, α-pinene, camphene, 3-carene, benzaldehyde and acetic acid (Fig. 3).

4. Discussion

It is known that many insect pests select their hosts based on visual and semiochemical stimuli (Cook et al., 2007a,b). Our field study results show that N fertilization affected pest host selection, although not in a simple linear manner. For both studied herbivorous insects, plants at moderate N treatments (N100–N140) were less attractive than plants treated with low or high N levels. Since the number of flowers and therefore the availability of suitable oviposition and feeding sites increased monotonically with added nitrogen, it could be suggested that host selection was possibly more affected by semiochemical stimulants than the flower abundance. However, additional laboratory experiments are needed to provide insight into the insect behavioural responses to altered volatile bouquets.

For M. aeneus females, the most preferred stage for oviposition is the bud stage with buds of only 2–3 mm in size (Borg, 1996; Nilsson, 1988). Therefore, the chemical components emitted by buds should be an important cue for the insects in locating B. napus plants. Measurements of VOCs in controlled environments detected a significant positive effect of N on acetic acid emission in the bud stage. Acetic acid has been demonstrated as product and intermediate in the biosynthesis of secondary metabolites in Brassica rapa (Abdel-Farid et al., 2007). Since the control plants were also less infested with the beetle larvae, acetic acid may be one of the cues for the females. Methyl salicylate emission was also detected at increased levels in plants treated with high N levels (N160) at both bud and flowering stages. This compound has been shown in numerous experiments to be attractants for sap-feeding (e.g. aphids, mites) and chewing (caterpillars, beetles) herbivores as well as for beneficial insect groups, including parasitic Hymenoptera (Kaplan, 2012; Rodriguez-Saona et al., 2011).

When looking at the statistical correlations between the mean abundance of larvae of M. aeneus and C. obstrictus in the field experiment and laboratory results on volatile compounds of B. napus buds and flowers, several compounds in addition to acetic acid and methylbenzoate were correlated (Table 3). A positive
correlation with mean *M. aeneus* larval number in flowers was detected for (3Z)-hexenyl acetate, camphene, 3-carene, limonene and indole emissions, and there were significant negative correlations with β-pinene, linalool, (E,E)-α-farnesene, methylbenzoate and benzaldehyde emissions. Depending on the direction of the correlation, these compounds could therefore be suggested as attractants or repellents for female pollen beetles.

**Female** *C. obstrictus* adults lay eggs into small developing siliques. They migrate to *B. napus* fields during the end of plants’ flowering stage and are attracted to both flower and leaf extract odour (Evans and Allen-Williams, 1992). In our study, several LOX compounds and monoterpenes as well as methyl salicylate and acetic acid were emitted from flowers in higher concentrations with increasing N levels. Among these compounds with enhanced emissions in our study, (3Z)-hexenol and methyl salicylate have been shown to attract weevils (Bartlet et al., 1993). Unfortunately, only α-pinene, camphene and β-pinene showed a significant, although weak correlation to insect oviposition activity in our study (Table 3). Although *C. obstrictus* life cycle is better synchronized with *B. napus* winter varieties than *M. aeneus* (Alford et al., 2003; Veromann et al., 2006a,b; Williams, 2010), the abundance of weevils in the field was very low during both study years. This could also be a reason for small or no differences detected in the abundance of this pest at different N treatments. The highest average percentage of infected siliques was only 14.3% for the two study years, which is clearly lower than the threshold of 26% that has shown to potentially cause significant yield losses (Free and Williams, 1978; Lerin, 1984). Therefore additional studies on years with more pests and possibly also more VOC collections may provide more information about this insect – host plant communication system.

The main searching cue in the spring for *B. napus* pests’ parasitoids is probably the odour of the crop (Williams and Cook, 2010). Jönsson et al. (2005) showed that the larval endoparasitoids of *M. aeneus* were attracted to yellow colour and odour from *B. napus* buds. However, parasitoids’ host search has generally two phases: localization of hosts’ habitat (plants) via plant volatiles from distance and thereafter localization of potential hosts (insects).
within the habitat (Godfray, 1994). In our study, the parasitoids’ host searching success for M. aeneus differed between years, but on average, it was the greatest in the control plots with the lowest number of buds and also with the smallest number of available insect hosts. Based on these results, we can speculate that even at close proximity, the host search might be affected by cues, both from plants and the insects. Based on these results, we can speculate that even at close proximity, the host search might be affected by cues, both from plants and the insects. Among VOCs in bud stage, methyl-salicylate and (Z)-hexenyl acetate, methyl salicylate has been found to attract several parasitoids, including parasitoids of cruciferous pests (James, 2005; James and Grasswitz, 2005; Kaplan, C. obstrictus there is very little information about parasitization of B. napus larvae was high at all treatments and strongly correlated with host availability. Although there is very little information about C. obstrictus parasitoids’ preferences, it is known that the main host searching cue for T. perfectus is the odour of brassica from the third instar of C. obstrictus (Dmoch and Rutkowska-Ostrowska, 1978; Dmoch, 1998; Williams, 2003). This is in concordance with our results as nitrogen treatment did not seem to have any impact on parasitoid abundance when separated from the impact of the host larvae. The results of this study demonstrate that when optimizing the disease control in B. napus, the plants may become more or less attractive for other biotic or abiotic stresses. As plant chemical defences can reduce the quality of the plant to the herbivore, it may be a signal of low host quality for the parasitoid (Godfray, 1994). Such combinations, however, are clearly very challenging to measure and interpret and need more attention in the future to help to understand these multi-level relationships in field conditions (Dicke and Baldwin, 2010; Kaplan, 2012; Mumm et al., 2008; Orre et al., 2010). In the case of many other fungal diseases (e.g. Söchting and Verreet, 2004), nitrogen fertilization can enhance fungal damage. However, in our study nitrogen fertilization significantly decreased the average A. brassicae occurrence on winter B. napus. Since the initial infection with A. brassicae occurred probably in the flowering stage, we compared the disease scores with VOCs from flowering plants. Several monoterpenes, green leaf volatiles and acetic acid were produced at higher levels at increased N dosages (Table 2). Since there is very few information about the impact of these compounds to fungal infections of plants, additional experiments are needed to determine if all of these can have antifungal activities. However, acetic acid has been experimentally shown to inhibit fungal growth (Delaquais et al., 1999; Kang et al., 2003) and therefore we suggest that increased acetic acid emission was one of the reasons for lower disease abundance of the fungal pathogen at greater nitrogen applications.

Confirming several previous studies (e.g. Cook et al., 2007b), our results show a clear difference in VOCs and their concentrations between the bud and flower stage of B. napus. Among the 19 detected compounds, there were only two – methyl salicylate and acetic acid – that were produced at increased levels at greater N levels both at bud and flowering stages. The effect of N nutrition on other compounds might have either been absent or detectable only in combination with other factors. It is clear that the bouquet of the volatiles is changing with plant development, but it is also clear that the effects of the surrounding ecosystem should be considered. For instance, it is possible, that after the first insects feed on the B. napus, the plants may become more or less attractive for other individuals depending on the nutrient availability in the soil (Olson et al., 2009). Additionally, infections with pathogenic or endophytic micro-organisms may influence the plant emissions. Since the emission of volatiles is previously shown to be highly variable under different biotic and abiotic stresses (Copolovic et al., 2011; Holopainen and Gershenzon, 2010; Niinemets, 2010; Toome et al., 2010), additional studies with insects feeding on the plants are needed to continue the studies of the effect of N on B. napus pests.

The results of this study demonstrate that when optimizing the N fertilization of B. napus plants, the changes in plants and their effect to other organisms in the cropping system should also be considered. Natural populations of parasitoids could rather efficiently control the pest populations and if favourable conditions are created, it would enable to cut back on the pest control cost. Last, but not least, optimized use of all chemicals in B. napus cropping

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### Table 3

Correlations between M. aeneus larval number and emission rates of volatiles from buds (bud stage), and C. obstrictus damaged pods and emission rates of volatiles from flowers (flower stage) in 2008–2009.

<table>
<thead>
<tr>
<th>Volatile organic compound</th>
<th>Meligethes aeneus</th>
<th>Conotrachelus obstrictus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2008</td>
<td>2009</td>
</tr>
<tr>
<td></td>
<td>$r_s$</td>
<td>$p$</td>
</tr>
<tr>
<td>(Z)-hexenyl acetate</td>
<td>0.19 ns</td>
<td>0.50 $&lt;0.001$</td>
</tr>
<tr>
<td>&quot;$\alpha$&quot;-pinene</td>
<td>-0.23 ns</td>
<td>-0.04 ns</td>
</tr>
<tr>
<td>camphene</td>
<td>0.19 ns</td>
<td>0.50 $&lt;0.001$</td>
</tr>
<tr>
<td>&quot;$\beta$&quot;-pinene</td>
<td>-0.38 $&lt;0.01$</td>
<td>-0.44 $&lt;0.001$</td>
</tr>
<tr>
<td>3-carene</td>
<td>-0.05 ns</td>
<td>0.35 $&lt;0.001$</td>
</tr>
<tr>
<td>Limonene</td>
<td>0.12 ns</td>
<td>0.52 $&lt;0.001$</td>
</tr>
<tr>
<td>Linalool</td>
<td>-0.36 $&lt;0.05$</td>
<td>-0.67 $&lt;0.001$</td>
</tr>
<tr>
<td>($E$/$E$)-&quot;e&quot;-farnesene</td>
<td>-0.38 $&lt;0.01$</td>
<td>-0.44 $&lt;0.001$</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>-0.34 $&lt;0.01$</td>
<td>-0.45 $&lt;0.001$</td>
</tr>
<tr>
<td>Indole</td>
<td>0.12 ns</td>
<td>0.32 $&lt;0.05$</td>
</tr>
<tr>
<td>Methylbenzoate</td>
<td>0.32 $&lt;0.05$</td>
<td>-0.68 $&lt;0.001$</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.19 ns</td>
<td>0.50 $&lt;0.001$</td>
</tr>
</tbody>
</table>

Spearman correlation coefficient ($r_s$) and its statistical significance ($p$) were calculated for nitrogen treatments N0, N80, N100 and N160 pooled.
systems would greatly decrease the environmental footprint of the production of this crop and therefore contribute to more environmentally friendly crop production and plant protection.

Acknowledgements

We thank Reelika Kevävi and Maris Saarn for field assistance and Jõgeva Plant Breeding Institute for crop husbandry. This study was supported by the Estonian Science Foundation (grants 7645, 8895), European Science Foundation EUROCORES project A-BIO- VOC, the Estonian Ministry of Science and Education grants SF1090065s07 and SF1070057s9; the capacity-building project P9003PKPK of the Estonian University of Life Sciences; and the European Commission through European Regional Fund (Center of Excellence in Environmental Adaptation) and European Social Fund (post-doctoral grant MJ1D4). We also thank the anonymous reviewers for their constructive and helpful criticism on an earlier version of the manuscript.

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