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ORIGINAL PAPER

Effect of nitrophenolates on pod damage caused by the brassica pod midge on the photosynthetic apparatus and yield of winter oilseed rape

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Abstract Oil seed rape (*Brassica napus* L.) is one of the most commonly grown crops in Central Europe, and the brassica pod midge (*Dasineura brassicae* Winn.) is one of the most important pests there. Insecticides against this pest applied during flowering may harm bees and other beneficial insects. The use of biostimulants such as nitrophenolates, which are not harmful to beneficial insects, can be an environmentally friendly way to control this pest. Nitrophenolates activate lignin synthesis in rape pods so the brassica pod midge is not able to penetrate pods. Nitrophenolates also regulate the efficiency of the photosynthetic apparatus, thus increasing yield. For these reasons nitrophenolates were tested in field conditions in Central Europe in 2005, 2007 and 2008. Nitrophenolates were applied on 10-m^2 plots, and their effect was compared to that of conventional insecticides. The number of damaged pods and yield parameters were assessed; the lignin content as well as photosynthetic rate was measured. Expression of genes related to lignin biosynthesis was examined in *Arabidopsis thaliana* L. The application of nitrophenolates decreased pod damage caused by the brassica pod midge. Expression levels of four genes related to lignin biosynthesis were increased after the application of nitrophenolates. The yield was higher in nitrophenolate-treated plots, which was attributed to an increase in the intensity of photosynthesis, higher chlorophyll content and improved chlorophyll *a* fluorescence parameters. The results showed that nitrophenolates have potential as a protective agent, but a further study is required. The application of nitrophenolates holds promise for reducing chemical input into the environment.

Keywords *Brassica napus* L. var. *oleifera* · *Dasineura brassicae* Winn syn. *Dasineura napi* Loew · Nitrophenolates · Lignin content · Efficiency of photosynthetic apparatus · Gene expression

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Introduction

Oilseed rape (*Brassica napus* L.) is an important crop in both food and nonfood sectors and plays a significant role as a raw material in various industrial processes. The annual world production of this crop currently amounts to approximately 400 million tonnes and is steadily increasing by 3–4 % per year. Oilseed rape seeds are harvested to obtain high quality oil for the food processing and chemical industries, and this crop accounts for approximately 12 % of total oilseed production (Dusser 2007).

The importance of oilseed rape as a source of industrial and nutritional oil has been growing worldwide; however, in some countries, an increase in acreage is accompanied by a dramatic, disproportionate increase in pesticide application (Zaller et al. 2008). Winter oilseed rape might suffer from unfavourable environmental conditions, such as severe winters, spring frosts, drought or pests infestation

(Budzyński et al. 2008; Krawczyk and Skoczyński 2008). Apart from fungal diseases, insect pests cause the greatest yield losses. Significant pest damage to winter oilseed rape is caused by the rape stem weevil (*Ceutorhynchus napi*, Gyllenhal), cabbage stem weevil (*Ceutorhynchus pallidactylus*, Marsham) and, in the case of buds and flowers, the pollen beetle (*Meligethes aeneus*, Fabricius). In Central Europe, the most damaging pests are those that attack oilseed rape pods in the BBCH 65–75 growth stages. Among these pests, the most significant are the cabbage stem weevil and the brassica pod midge (Skuhravý and Skuhravý 1960; Evans and Allen-Williams 1989; Bromand 1990).

Two insecticides based on pyrethroid and neonicotinoid are currently used for protection of pods against insect infestation. These insecticides are applied during the period of pod development, when the female insects of pests lay their eggs (Pavela et al. 2007). However, at that time, large quantities of other non-targeted insect species, such as pollinators and natural enemies of the pests from the order Hymenoptera (e.g. *Omphale* spp., *Tetrastichus* spp., *Entedon* spp., *Aprostocetus* spp. and *Chrysocharis* spp.) are also present in the field (Šedivý 1983; Goulet and Huber 1993; Alford 2003; Williams 2003). For these and other reasons, such as the appearance of pest resistance to active substances as well as the environmental and health risks associated with insecticide residues (Elzen and Hardee 2003), there is growing pressure to develop and use new, environmentally safe pest control measures.

In recent years, farmers have been using biostimulants (syn. biostimulators) (Budzyński et al. 2008; Przybysz et al. 2008) to protect oilseed rape against stress factors. According to a definition provided by the European Biostimulants Industry Council (EBIC), biostimulants include diverse formulations of compounds, substances and microorganisms that are applied to plants or soils to improve crop vitality, yield, yield quality and tolerance to stress (EBIC 2011). Biostimulants also differ from crop protection products because they act only on plant vitality and do not have any direct actions against pests or diseases. Biostimulants can be used as an alternative to synthetic insecticides (Conrath et al. 2002). Nitrophenolates fall into the category of substances that stimulate natural plant vitality and stress tolerance (Gawrońska et al. 2008; Przybysz et al. 2010).

The nitrophenolates used in this study include sodium para-nitrophenolate (PNP), sodium ortho-nitrophenolate (ONP) and 5-nitro-guaiacol sodium (5NG). These compounds easily penetrate into plants, where they are metabolised. Nitrophenolates participate in a number of metabolic processes (Stutte and Clark 1990); for example, they increase cytoplasmic streaming (Wilson and Kaczmarek 1993) or the concentration of endogenous auxins (which ensures enhanced activity) (Djanaguiraman et al. 2004a, 2005a; Stutte and Clark 1990), promote growth and generative development (Djanaguiraman et al. 2004a, 2005a, b; Gruszczyk and Berbeć 2004; Budzyński et al. 2008; Gawrońska et al. 2008; Przybysz et al. 2008), stimulate nutrient uptake (Stutte et al. 1987) and enhance photosynthetic activity (Gawrońska et al. 2008; Przybysz et al. 2008). A plant's reaction to stress is thereby often enhanced by faster and more effective activation of defence mechanisms. The ability to overcome stress improves the health of plants and boosts their growth, which results in an increase in yield and improvement in product quality (Babuška 2004). The positive effect of nitrophenolates on crop growth, yield and yield quality has been recorded for cotton plants (Bynum et al. 2007), oilseed rape (Babuška, 2004; Budzyński et al. 2008; Przybysz et al. 2008) and sugar beets (Cerny et al. 2006) as well as medicinal plants such as feverfew (*Chrysanthemum parthenium*) (Gruszczyk and Berbeć 2004) and the common motherwort (*Leonurus cardiaca*) (Kieltyka-Dadasiewicz and Berbec 2007).

Brassica species are closely related to *Arabidopsis thaliana*, which makes it a very good model system for the *Brassicaceae* family members. *A. thaliana* and *B. napus* share about 85 % exon sequence similarity at the nucleotide level (Cavell et al. 1998). The complete genome of *A. thaliana* has been sequenced (Arabidopsis Genome Initiative 2000), assembled and used for analysis of functions of genes related to other crops such as *B. napus*, for instance in case of genes related to seed development (Girke et al. 2000) and oil content (Fu et al. 2009; Guan et al. 2012). It seems to be appropriate to take the option of using the *A. thaliana* microarray platform for checking whether some changes in oilseed rape plants have a molecular background.

General knowledge of nitrophenolates is already quite extensive, and the biological basis of their mode of action is known (Gawrońska et al. 2008; Djanaguiraman et al. 2010; Yaneva and Masheva 2010). However, little is known about how their application can influence the yield of oilseed rape. To the best of our knowledge, data regarding their effects on the damage caused by pod larvae are not available in the literature. We expected that nitrophenolate application could activate plant growth (including lignin synthesis) and pods were less vulnerable to damage by pod midge. Nitrophenolates could also enhance photosynthesis activity and positively affect defence mechanisms; all these mechanisms should lead to yield increase of oil seed rape. Therefore the objective of this study was to determine the effect of nitrophenolate application on (1) pod damage by brassica pod midge larvae infestation, (2) efficiency of the photosynthetic apparatus and (3) yield and yield-related parameters of winter oilseed rape grown under field conditions. We also examined

Table 1 Localities used for experiments

Locality	Altitude (m above sea level)	GPS	50-year av. ann. air temp. (°C)	50-year average rainfall (mm)	Soil type	Experiment
1. Humpolec	525	49°32′88″N 15°32′99″E	6.54 12.7 (during growing season)	667 400–450 (during growing season)	Gleyed and loamy sand (pH 6.0)	1–3 (Pod damage by brassica pod midge, yield assessment)
2. Nechanice	535	50°14′28″N 15°37′13″E	8.8	597	brown and loamy (pH 6.7)	1–3 (Pod damage by brassica pod midge, yield assessment)
3. Uhříněves	290	50°2′6″N 14°37′4″E	8.3	575	brown clay soil (pH 7.0)	1–3 (Pod damage by brassica pod midge, yield assessment)
4. Chylice (Poland)	105	22°33′25″N 52°05′71″E	7.8 12.8 (during growing seasons)	591.8 (30-year av.) 448 (during the growing seasons)	black degraded soil composed of loamy sand	4, 5 (Photosynthetic apparatus efficiency, yield parameters)

whether this preparation altered lignin content and the expression of genes relative to lignin biosynthesis and metabolism.

Materials and methods

Experiments were carried out in three localities in the Czech Republic and one locality in Poland (Table 1). In the experiments undertaken in the Czech Republic, an attempt was made to determine the effects of nitrophenolates on (1) pod damage caused by the brassica pod midge (*Dasineuranapi*, Loew), (2) the yield of oilseed rape *cvs.* Pronto (2005) and Lisek (2007 and 2008) and (3) the lignin content in *cv.* Oponent grown in 2009.

Experiments in Poland were designed to investigate (4) the efficiency of the photosynthetic apparatus and intensity of transpiration, and (5) plant growth, biomass accumulation, yield and yield parameters of oilseed rape *cv.* Lisek grown in 2007 and 2008. Additionally, these studies examined (6) gene expression levels in *Arabidopsis thaliana* L. grown in growth chambers in 2008.

Pod damage caused by brassica pod midge, yield of oilseed rape plants and lignin content

Experiments 1–3 were conducted in complete randomised blocks in four replicates at all localities, the plants were grown at 12.5 cm × 14 cm spacing on 10-m^2 plots. Agrotechnical practices (sowing date and rate, spacing, fertilising, etc.) were the same for each plot according to the recommendation for this species with respect to the location's weather conditions. Each plot (including controls) was treated by herbicide (clomazone) in the fall; pesticides (chlorpyriphos and cypermethrin) against weevils were applied in April. Pesticides against pollen beetle and pod midge were not used for obvious reasons.

Experiment 1: Pod damage by brassica pod midge

Pod damage by brassica pod midge was monitored according to the EPPO method (OEPP/EPPO 1998). To determine the pod damage caused by *Dasineura brassicae* larvae at BBCH 75 (on the 15–18th days after treatment with the preparations listed below), at least 100 pods per plot were collected from 10 plants that were assessed separately (five pods per plant were taken from primary branches and five were taken from secondary branches). The pods were selected using a standard method (i.e. starting from the top of the raceme flower head and moving downward every third pod). Undeveloped pods were excluded.

Experiment 2: Crop yield assessment

To determine crop yield, all plants from every plot were harvested in August using a Seedmaster Advanced, and the undamaged seeds were air-separated and weighed. The yield was calculated in kg ha^{-1} and adjusted to a fixed moisture level (12 %).

Experiment 3: Pod lignin analysis

The lignin content of the pods was analysed in 2009. Sampling points were established at a distance of 40 cm from one another along a diagonal line across the plots. From these points, 50 small pods (with a length up to 2 cm), 50 medium pods (2–5 cm) and 50 large pods (over

5 cm) were collected. Immediately after collection, the samples were freeze-dried and subjected to analysis. Acid-detergent lignin was determined using the procedures of Goering and Van Soest (1990), and the method was modified using the filter bag system (Vogel et al. 1999).

Efficiency of the photosynthetic apparatus, yield and yield parameters

In these experiments, the effect of nitrophenolates on plant growth, efficiency of the photosynthetic apparatus and yield of oilseed rape (*Brassica napus* L. var. *oleifera cv.* Lisek) was evaluated. Experiments were conducted at WULS-SGGW Experimental Farm at Chylice (near Warsaw, Poland) in complete randomised blocks in four replicates (plots of 18 and 14.4 m^2 in 2007 and 2008 respectively). The seeds were sown at a spacing of 30 × 6.5 cm. Routine agricultural practices recommended for this species and location were employed. In the 2007 growing season, some of the examined plants were grown in 25-L pots placed in the soil of particular plots.

Experiment 4: Efficiency of the photosynthetic apparatus and intensity of transpiration

One week after the first nitrophenolate application, the following parameters were measured in vivo: (1) intensity of photosynthesis, (2) stomatal resistance (LICOR 6200 Photosynthesis System, Lincoln, NE, USA), (3) total chlorophyll content (CCM-200, OPTI-SCIENCES, USA) and (4) maximum quantum efficiency of photosystem II (Fv/Fm) and performance index (an indicator of vitality, PI) [based on the fluorescence of chlorophyll *a* measurements (Handy PEA, Hansatech, UK)]. The transpiration rate was recorded (LICOR 6200 Photosynthesis System, Lincoln, Nebraska, USA) simultaneously with the photosynthetic apparatus efficiency. The measurements were performed for 8 (2007) and 5 (2008) consecutive weeks.

Experiment 5: Plant growth, biomass accumulation, yield and yield parameters

At harvest, the following parameters were measured: (6) plant height, (7) number of primary branches and pods per plant as well as number of seeds per pod, (8) accumulation of biomass and (9) yield.

Experiment 6: Changes in gene expression related to lignin biosynthesis and metabolic processes

These experiments were performed at WULS-SGGW in 2008 using three biological replicates with dye swapping. For monitoring changes in gene expression levels, cDNA microarray technology was employed (*Arabidopsis thaliana* Genome Oligo Set, Version 3.0, OPERON). *Arabidopsis thaliana* L. Col-4 seeds (Round Rock, TX, USA) were sown onto multiplates filled with substrate mixed with sand at a proportion of 2:1 (v/v). Uniform, 6-week-old seedlings were transplanted to pots (Ø 10 cm) and grown in a growth chamber (Simez Control s.r.o., Vsetin, Czech Republic) at 22/18 °C with a photoperiod of 8/16 h day/night. The photosynthetic photon flux density (PPFD) of PAR was 250–280 μmol m^{-2} s^{-1} at the plant's level, and the relative humidity was 60 %. One week after transplanting, nitrophenolates were applied as a foliar spray at a concentration of 0.1 % (v/v) once. Leaf samples were collected 24 h after treatment and stored at −80 °C until they were used for RNA isolation. Total RNA was isolated with TRIZOL Reagent (Invitrogen), digested with DNase (Fermentas) and cleaned with the RNeasyMinEluteCleanup Kit (Qiagen). The RNA quality was determined using a Bioanalyzer (Agilent Technologies) and the Agilent RNA 6000 NanoLabChip Kit. Total RNA was reverse transcribed into cDNA using reverse transcriptase, and the cDNA was labelled with Alexa 555 and Alexa 647. The labelled cDNA was then hybridised to microarrays with a HybrArray 12 (PerkinElmer Precisely), and the results were scanned using a ScanArray Express HT (PerkinElmer Precisely). After standardisation and normalisation of the excited dye signals, genes with altered expression were determined. Genes that showed at least twofold increase in expression or 0.5-fold decrease in expression due to nitrophenolate treatment were marked as genes with higher and lower expression respectively. Genes with altered expression levels were compared with the available databases (TAIR, MIPS, NCBI and KEGG [Kyoto University Encyclopaedia]) to determine their functions.

Chemical preparations

In field experiments 1–3 nitrophenolates were applied at the beginning of flowering (BBCH 60-61) at doses of 0.6 g ha^{-1} 2-methoxy-5-nitrophenolate Na, 1.2 g ha^{-1} 2-nitrophenolate Na and 1.8 g ha^{-1} 4-nitrophenolate Na (Fig. 1). Nitrophenolate doses were applied according to the manufacturer's recommendations (Atonik, Asahi Chemical Co., Ltd., Ikomagun Nara, Japan). In all locations, the effects of nitrophenolates were compared with those of two synthetic insecticides: one based on lambda-cyhalothrin at a dose of 7.5 g of active ingredient ha^{-1} (Karate Zeon 5CS, Syngenta International AG, Switzerland) and a second based on acetamiprid at a dose of 24.0 g of active ingredient ha^{-1} (Mospilan 20SP, Nipon Soda, Ltd., Japan). All doses were based on the

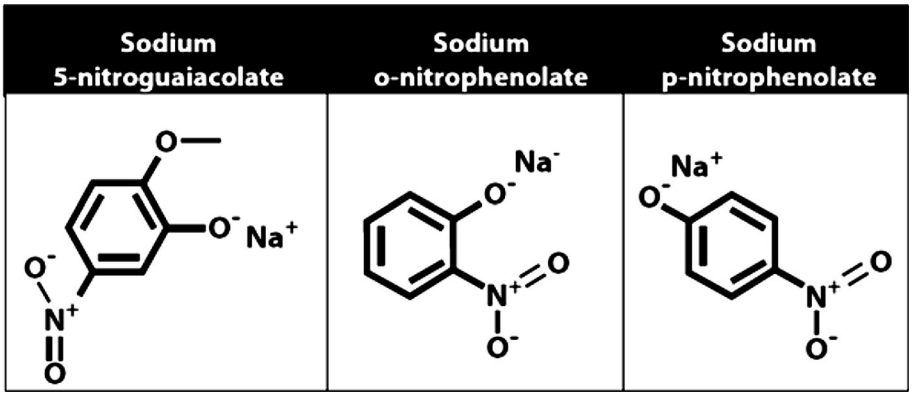

Fig. 1 Structural formulas of used nitrophenolates

manufacturer's recommendations. The control plots were not treated by any chemical preparation or water; other agrotechnical practices were the same as in the experimental plots.

In experiments 4 and 5 examining the efficiency of the photosynthetic apparatus, yield and yield parameters, nitrophenolates were applied in the spring in the form of Atonik (Asahi Chemical Co., Ltd., Ikomagun Nara, Japan) as a single (BBCH 29-31) or double (BBCH 29-31 and BBCH 51) foliar spray (300 l ha^{-1}) at a concentration of 0.2 or 0.4 % respectively. The concentrations of active ingredients were 0.6 g ha^{-1} 2-methoxy-5-nitrophenolate Na, 1.2 g ha^{-1} 2-nitrophenolate Na and 1.8 g ha^{-1} 4-nitrophenolate Na in the single treatment; twice as much of each active ingredient was present in the double treatment.

Statistical analysis

Field experiments 1–3 were conducted with four replicates in a complete randomised block design. The data were subjected to analysis of variance (ANOVA) using Statgraphics Plus 4.0. Pod damage is expressed as a percentage compared with the untreated control (the control is set to 100 %; pod damage, yield, etc., vary at different localities; therefore transfer to percentage made all data comparable). Differences between the combinations were estimated using the Tukey HSD test at the 95 % confidence level. The data from the insecticide trials were analysed separately for each year, and the percentage data were subjected to an arcsin, square-root transformation before analysis (according Pavela et al. 2009).

Field experiments 4–6 were made in four plots. Depending on the parameter, presented data are the mean of 20 or 36 replications (mentioned in the table and figure captions). The data are presented as the mean ± SE (where indicated). The data were subjected to one-way ANOVA. Differences between the combinations were evaluated by LSD (Student's t test) at $\alpha = 0.05$.

Results

Effect of nitrophenolates on pod damage by brassica pod midge, lignin content and yield

Experiment 1: Pod damage by brassica pod midge

The number of damaged pods in nitrophenolate-treated plants was always lower than in the control plants, and the biological efficiency of this preparation was either at the level of the insecticide acetamiprid or, most often, higher than that of acetamiprid. The pod damage in plots treated with a tank-mix (nitrophenolates and lambda-cyhalothrin) was the same as that in plots treated with nitrophenolates alone. It was evidently lower than what was observed with control plants (Table 2). The efficiency of nitrophenolates differed between locations and years of study, being the highest in 2007 (at Humpolec) and 2005 (at Nechanice) and the lowest in 2005 (at Humpolec) and 2007 (at Nechanice) (Table 2).

Experiment 2: Crop yield assessment

Compared with the untreated control plots, there were significant yield increases in Nechanice [0.7 t ha^{-1} (20 %) in 2005 and 0.4 t ha^{-1} (12.9 %) in 2007] and in Humpolec [0.5 t ha^{-1} (17.2 %) in 2008]. The yield of plants treated with nitrophenolates was also higher than in plots treated with acetamiprid insecticide. The exception was in Humpolec in 2007 and 2008, where the yield was at the same level or slightly lower. It is worth noting that the effect of nitrophenolates was greater in Nechanice than in Humpolec in every year of the study. In some cases, the tank-mix application (nitrophenolates and lambda-cyhalothrin) resulted in a yield increase up to 14.6 % compared with the application of nitrophenolates alone (e.g. Humpolec in 2007); however, in most cases, no differences were noted between these two treatments or nitrophenolates alone were more effective (Table 3).

Table 2 Effect of tested preparations on pod damage by Brassica pod midge in oilseed rape plants grown in two locations and three vegetation seasons

Combination	2005		2007		2008	
	Humpolec[1]	Nechanice[2]	Humpolec[3]	Nechanice[4]	Humpolec[5]	Nechanice[6]
	% of damaged pods					
Control	3.8 ± 0.6a*	2.4 ± 0.8a	1.3 ± 0.1a	10.2 ± 1.5a	3.3 ± 0.4a	4.9 ± 0.5a
Acetamiprid	1.6 ± 0.2b	1.9 ± 1.0ab	0.6 ± 0.1b	6.0 ± 1.1bc	2.1 ± 0.2ab	3.1 ± 0.8ab
Nitrophenolates	1.5 ± 0.2b	1.6 ± 0.1b	0.6 ± 0.1b	4.5 ± 0.9c	1.1 ± 0.2c	2.1 ± 0.5b
Nitrophenolates + γ-cyhalothrin	1.5 ± 0.2b	1.4 ± 0.3b	0.5 ± 0.1b	7.2 ± 0.6b	1.5 ± 0.2bc	2.1 ± 0.2b

Data are mean ± SE, $n = 4$ plots with 100 pods in each

* Data in columns followed by the same letter do not differ significantly as based on the HSD index of the Tukey test at confidence level 95 %. (ANOVA $^{1}F = 39.97$, $^{1}P = 0.0000$; $^{2}F = 1{,}6940$, $^{2}P = 0.2211$; $^{3}F = 32.33$, $^{3}P = 0.00003$; $^{4}F = 19.73$, $^{4}P = 0.00006$; $^{5}F = 55.37$, $^{5}P = 0.00000$; $^{6}F = 19.72$, $^{6}P = 0.0000$, $^{1-6}df = 12$)

Table 3 Effect of tested preparation on yield (t/ha) of oilseed rape plants grown in two locations and three vegetation seasons

Combination	2005		2007		2008	
	Humpolec[1]	Nechanice[2]	Humpolec[3]	Nechanice[4]	Humpolec[5]	Nechanice[6]
	Yield (t ha^{-1})					
Control	4.9 ± 0.2a	3.5 ± 0.2b	4.7 ± 0.2a	3.1 ± 0.1b	2.9 ± 0.2b	4.0 ± 0.1a
Acetamiprid	5.1 ± 0.2a	4.1 ± 0.4ab	4.9 ± 0.2a	3.4 ± 0.1a	3.4 ± 0.1a	4.1 ± 0.1a
Nitrophenolates	5.3 ± 0.2a	4.2 ± 0.1a	4.8 ± 0.2a	3.5 ± 0.1a	3.4 ± 0.2a	4.3 ± 0.1a
Nitrophenolates + γ-cyhalothrin	5.1 ± 0.1a	4.1 ± 0.1a	5.5 ± 1.5a	3.5 ± 0.1a	3.6 ± 0.1a	4.2 ± 0.1a

Data are mean ± SE, $n = 4$ plots

[1] Data in columns followed by the same letter do not differ significantly as based on the HSD index of the Tukey test at confidence level 95 %. (ANOVA $^{1}F = 1.98$, $^{1}P = 0.1706$; $^{2}F = 3.92$, $^{2}P = 0.3658$; $^{3}F = 0.74$, $^{3}P = 0.5468$; $^{4}F = 5.12$, $^{4}P = 0.0289$; $^{5}F = 9{,}081$, $^{5}P = 0{,}0021$; $^{6}F = 4.24$, $^{6}P = 0.0293$, $^{1-6}df = 12$)

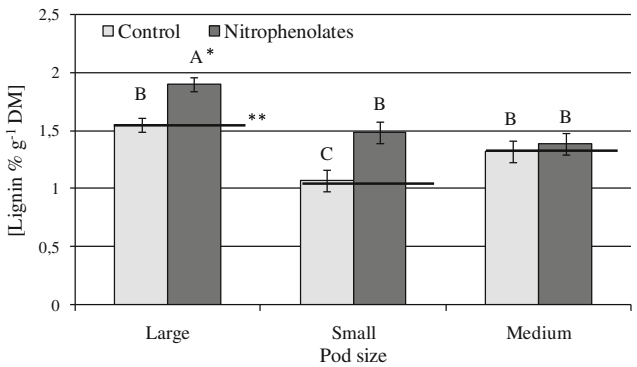

Fig. 2 Lignin content in pods of oilseed rape *cv*. Oponent plants grown under field conditions treated by nitrophenolates in the 2009 vegetation season. Data are mean ± SE, $n = 4$ (with 50 pods in each replication and every pod size). **Bars* marked with the *same letter* do not differ significantly as estimated by HSD values of the Tukey test at confidence level 95 %. ***Horizontal lines* refer to the level recorded for not treated plants (control)

Experiment 3: Pod lignin analysis

Lignin content in small and large pods treated by nitrophenolates was higher by 40 and 22 % respectively, than in the control pods, (Fig. 2). The increase in lignin content in medium pods was lower (by 5 %). The lignin content in the small pods after nitrophenolate application corresponded with the lignin content of the large pods, which were not treated with nitrophenolates (Fig. 2).

Plant growth, efficiency of the photosynthetic apparatus, yield and yield parameters

Experiment 4: Efficiency of the photosynthetic apparatus and intensity of transpiration

In the 2007 vegetative season, the intensity of photosynthesis was higher in both single- and double-treated plants (1–22 %). This effect was observed for up to 7 weeks after the first treatment (Fig. 3a). In 2008 growing season, the positive influence of nitrophenolates on the intensity of photosynthesis lasted for 4 weeks. During this period, measured values were higher than in the control plants by 3.5–20.3 % (Fig. 3b). From the beginning of the 5th week, the intensity of photosynthesis was equal to or lower than the level photosynthesis of non-treated plants. With few exceptions, total chlorophyll content in both growing

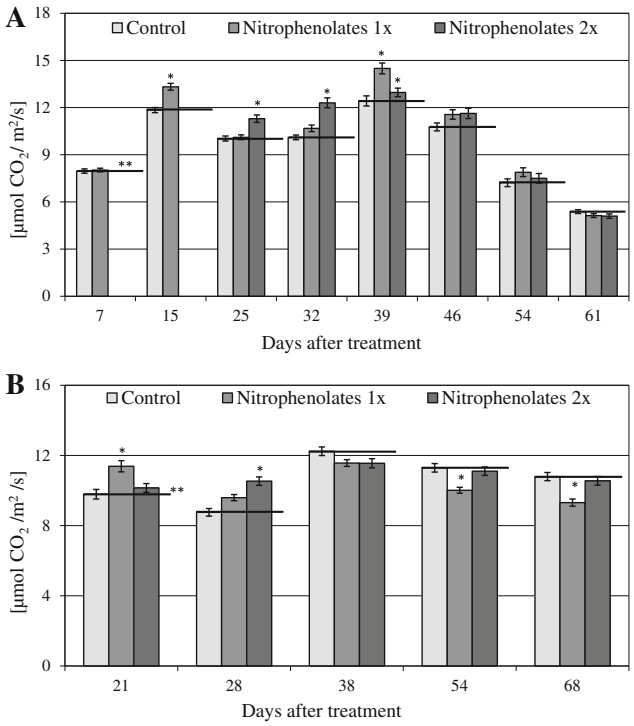

Fig. 3 Intensity of photosynthesis of oilseed rape *cv.* Lisek plants grown under field conditions treated by nitrophenolates in the 2007 (**a**) and 2008 (**b**) vegetation season. Presented data are mean ± SE, $n = 24$ (2007) and 36 (2008). **a** *Values differing significantly as estimated by LSD values of Student's *t* test at confidence level 95 %. ****Horizontal lines** refer to the level recorded for non-treated plants (control).**b** *Values differing significantly as estimated by LSD values of Student's *t* test at confidence level 95 %. ****Horizontal lines** refer to level recorded for non-treated plants (control)

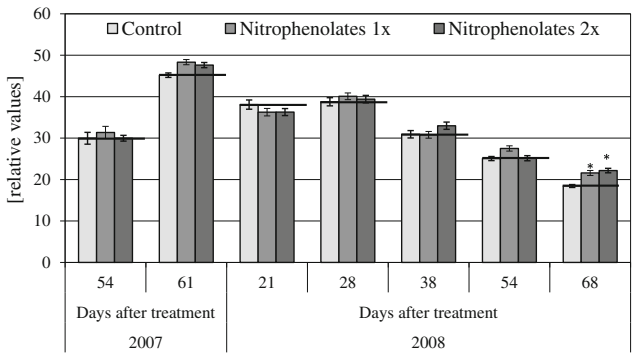

Fig. 4 Chlorophyll content in oilseed rape *cv.* Lisek plants grown under field conditions treated by nitrophenolates in the 2007 and 2008 vegetation seasons. Presented data are mean ± SE, $n = 36.4$. Selected parameters of chlorophyll *a* fluorescence:Fv/Fm (maximum quantum efficiency of Photosystem II) and PI (performance index, vitality indicator) for oilseed rape *cv.* Lisek plants grown under field conditions treated by nitrophenolates in the 2007 (**a**) and 2008 (**b**) vegetation season. Presented data are mean ± SE, $n = 24$. *Values differing significantly as estimated by LSD values of Student's *t* test at confidence level 95 %. ****Horizontal lines** refer to level recorded for non-treated plants (control)

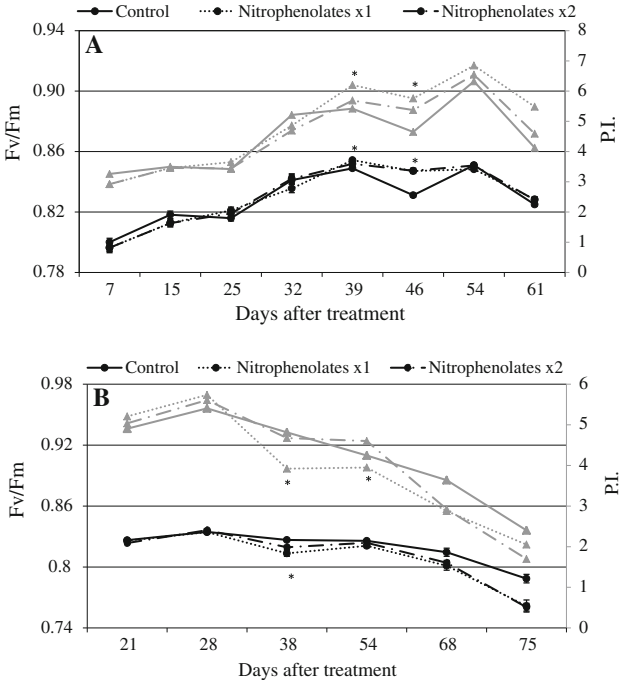

Fig. 5 **a** *Values differing significantly as estimated by LSD values of Student's *t* test at confidence level 95 %. **b** *Values differing significantly as estimated by LSD values of Student's *t* test at confidence level 95 %

seasons was slightly higher in nitrophenolate-treated plants. This was especially evident in the last measurements (Fig. 4).

In 2007, chlorophyll *a* fluorescence, Fv/Fm or PI until the 39th day after the first treatment was the same in control and treated plants. Between the 36th and 39th day after the first nitrophenolate treatment, a spring frost (–4.2 °C) occurred in Chylice (Fig. 5a). Following the frost, a decrease in the Fv/Fm was recorded in the control plants, whereas Fv/Fm did not change in treated plants. A similar pattern was noted for Fv/Fo (a parameter describing the oxygen-evolving complex, data not shown). Additionally, the PI values in control plants were lower after the frost, whereas these values were higher in treated plants and remained as such for all four measurements following the frost (Fig. 5a). In the 2008 growing season, during the first 10 weeks after biostimulant application, the values of Fv/Fm were similar in nitrophenolate-treated and untreated plants (Fig. 5b); this was also true for Fv/Fo (data not shown). However, from the 10th week after biostimulant application, decreases were recorded in these parameters. Nitrophenolates exerted positive effects on PI only in the first two measurements (Fig. 5b). Uniform trends were not observed in stomatal resistance or transpiration rate in either year; however, a slight decrease in stomatal resistance and a slight increase in transpiration rates were observable (Table 6).

Table 4 Effect of nitrophenolates on selected parameters of field grown oilseed rape *cv.* Lisek plants measured at harvest

Combination	Height (cm/plant)	(Number/plant)		(Number/pod)
		Laterals	Pods	Seeds
Growing season 2007*				
Control	108.90 ± 1.38a***	5.55 ± 0.27a	100.45 ± 5.50a	15.92 ± 0.14a
Nitrophenolates 1×**	118.89 ± 1.81b	5.42 ± 0.21a	104.58 ± 5.17a	16.06 ± 0.13a
Nitrophenolates 2×**	111.28 ± 1.43a	5.22 ± 0.24a	100.56 ± 7.27a	16.37 ± 0.15a
Growing season 2008				
Control	162.10 ± 1.29a	10.05 ± 0.25a	253.00 ± 8.50a	25.46 ± 0.18a
Nitrophenolates 1×	161.63 ± 1.16a	11.31 ± 0.26a	273.63 ± 11.87a	25.98 ± 0.42a
Nitrophenolates 2×	160.05 ± 2.13a	11.00 ± 0.23a	250.08 ± 8.91a	25.65 ± 0.71a

Presented data are mean ± SE, $n = 20$

* In 2007 growing season plants were grown in 25-l pots placed in the soil of plots of particular combination

** 1× and 2× refer to single or double spray respectively

*** Values differing significantly at $\alpha = 0.05$ as determined by LSD of Student's t test

Experiment 5: Plant growth, biomass accumulation, yield and yield parameters

During the 2007 season, the application of nitrophenolates had a positive effect on most of the measured parameters (Table 4). Plants exposed to single and double treatments were 9 and 2 % taller respectively. These plants were also more advanced in development and particularly in generative parts, producing more pods per plant (up to 4.1 %) and more seeds per pod (between 0.9 and 2.8 %). On the other hand, nitrophenolate-treated plants produced fewer primary laterals (between 2.5 and 6 %) (Table 4). In 2008, plant height was the same. Regardless of the number of applications, nitrophenolates increased the number of primary laterals (between 4.8 and 7.8 %) and the number of seeds per pod (between 0.7 and 2 %). Single nitrophenolate treatment had a positive effect on pod number, which was higher than in the control by 8.1 % (Table 4).

During the 2007 season, nitrophenolate-treated plants accumulated more biomass (Table 5). The total fresh weight of single- and double-treated plants increased by more than 12 %, and their dry matter proportions were 23.6 and 11.9 % respectively. Additionally, the fresh weight and dry matter of pods with seeds were increased in treated plants; both were higher in single-treated plants. The fresh weight and dry matter of the main stem were always higher in plants treated with a biostimulant. Double nitrophenolate application increased biomass accumulation in the laterals (Table 5). In the 2008 season, only a single treatment with nitrophenolates had a positive effect on biomass accumulation. The application of the biostimulant increased fresh weight and dry matter of the aboveground parts (4.3 and 2.5 % respectively), pods with seeds (2.4 and 4.7 % respectively) and main stem (10.9 and 3.8 % respectively). Regardless of the number of applications, nitrophenolates had a negative influence on the weight of the primary laterals (Table 5, 6).

Seed yield was affected by nitrophenolate application as well (Table 5). In 2007, the seed yield of single-treated plants exceeded the yield of control plants by 35 %. On the contrary, when nitrophenolates were applied twice, no positive effect was recorded. In the 2008 vegetative season, the effect of the biostimulant on seed yield was evidently smaller; additionally, only the single treatment increased seed yield (3.6 %). In the case of the double treatment, a reduction (12.2 %) in seed yield was recorded (Table 5).

Experiment 6: Changes in gene expression related to lignin biosynthesis and metabolic processes

The application of nitrophenolates to *A. thaliana*, which is taxonomically close to oilseed rape and is a model plant for molecular studies, resulted in changes in the gene expression profile (Table 7). Of the 26,173 protein-coding genes represented on the microarray, the expression levels were altered in 3,425 genes at 24 h after nitrophenolate treatment. In these altered genes, 97.5 % (3,339) were upregulated and only 2.5 % (86) were downregulated. In the upregulated genes, four were related to lignin, and their expression levels increased between 2.01 and 17.56 times under nitrophenolate treatment when compared with the control. Based on a comparison with the available databases, the functions of these of these four genes were confirmed to be related to lignin biosynthesis and metabolic processes (Table 7).

Table 5 Effect of nitrophenolates on fresh weight, dry matter of above ground part and yield of field grown oilseed rape *cv.* Lisek plants recorded at harvest

Measured parameter	Combination	(g/plant)				
		Above-ground part	Pods with seeds	Main stem	Laterals	Yield of seeds
Growing season 2007*						
Fresh weight	Control	37.42 ± 1.96a***	14.00 ± 0.85a	19.69 ± 1.02a	3.74 ± 0.37a	
	Nitrophenolates 1×**	42.10 ± 2.79a	16.15 ± 1.17a	22.36 ± 1.36a	3.59 ± 0.34a	
	Nitrophenolates 2×**	42.18 ± 2.78a	15.94 ± 1.97a	21.21 ± 1.43a	5.02 ± 0.50a	
Dry matter	Control	9.60 ± 0.51a	4.35 ± 0.27a	4.21 ± 0.20a	1.03 ± 0.10a	2.29 ± 0.16a
	Nitrophenolates 1×	11.87 ± 0.66a	5.52 ± 0.36a	5.33 ± 0.26a	1.02 ± 0.08a	3.08 ± 0.23a
	Nitrophenolates 2×	10.75 ± 0.67a	4.60 ± 0.39a	4.93 ± 0.26a	1.21 ± 0.10a	2.29 ± 0.24a
Growing season 2008						
Fresh weight	Control	147.86 ± 6.24a	59.86 ± 2.60a	62.84 ± 2.55a	25.15 ± 1.71a	
	Nitrophenolates 1×	154.26 ± 5.68a	61.28 ± 2.59a	69.69 ± 2.32a	23.28 ± 1.31a	
	Nitrophenolates 2×	136.82 ± 5.44a	50.89 ± 2.01a	63.58 ± 2.32a	22.36 ± 1.58a	
Dry matter	Control	68.46 ± 2.50a	42.12 ± 1.55a	15.75 ± 0.53a	10.59 ± 0.58a	24.69 ± 0.94a
	Nitrophenolates 1×	70.18 ± 2.19a	44.11 ± 1.44a	16.34 ± 0.37a	9.73a ± 0.46a	25.57 ± 1.03a
	Nitrophenolates 2×	62.55 ± 2.42a	38.03 ± 1.55a	15.33 ± 0.45a	9.18 ± 0.48a	21.93 ± 0.93a

Presented data are mean ± SE, $n = 20$

* In the 2007 growing season plants were grown in 25-l pots placed in the soil of plots of particular combination

** 1× and 2× refer to single or double spray respectively

*** Values differing significantly at $\alpha = 0.05$ as determined by LSD of Student's t test

Discussion

Findings of this study clearly show that the application of nitrophenolates positively affects field-grown oilseed rape plants, in terms of both protection against stressors and stimulation of vital processes under near-optimal conditions. A reduction in the pod damage caused by brassica pod midge was recorded in each year of the study at both locations. Moreover, the protective effect was either on the same level or even greater when compared with the application of acetamiprid insecticide or in combination with lambda-cyhalothrin. This study is the first to evaluate nitrophenolates as protective agents against pod damage caused by the brassica pod midge; thus, it is too early to conclude that some insecticides should be replaced by nitrophenolates. However, the results obtained here showed that nitrophenolates have significant potential as protective agents, and further study is warranted. Presently there is no evidence suggesting that nitrophenolates are toxicto pollinators, mammals, or humans and plants (EFSA Scientific Report 2008; EPA 2001) and soil and water are residue-free shortly after application (Djanaguiraman et al. 2004b).

The reduction in pod damage by the brassica pod midge coincided with an increase in the pod lignin content, and the lignin level in young pods was similar to that in older and larger pods. *Dasineura brassicae* lays eggs on young pods, and larvae feed on these pods during development (Pavela et al. 2009). Additionally, the increased lignin content in pods corresponds well with significant upregulation of four genes related to lignin biosynthesis and metabolism. Although gene expression was monitored using *A. thaliana* plants, we believe that such a comparison can be made because these species are taxonomically close (Cavell et al. 1998; Girke et al. 2000; Guan et al. 2012).

In all plots, plants treated with nitrophenolates had reduced pod damage and thus produced better yields. It should be noted that the reduction of pod damage and the yield increase differed depending on location and year, as the specific environmental conditions during particular years and at particular locations influenced the effectiveness of the examined biostimulant. Although the differences in yield between treated plots versus untreated controls were not always significant, a trend toward higher yields in nitrophenolate-treated plants was clear. The yield increase as an effect of nitrophenolate treatment was similar or greater than that noted in plots treated with acetamiprid insecticide or a combination of nitrophenolates and lambda-cyhalothrin.

In one of the experiments (no. 5), nitrophenolate application had a different effect on the number of primary laterals between years. Nitrophenolate-treated plants produced fewer laterals in 2007, but the production was greater the following year. This might be caused by variability of vegetation seasons among years. Spring frost in 2007 caused mortality of laterals, but application of nitrophenolates induced higher accumulation of biomass

Table 6 Effect of nitrophenolates on stomatal resistance and transpiration rate of oilseed rape *cv.* Lisek plants grown under field conditions in 2007 and 2008 vegetation season

Measured parameter	Combination	Days after first spray							
		7	15	25	32	39	46	54	61
Growing season 2007*									
Stomatal resistance (s cm^{-1})	Control	4.60a ± 0.08	2.83b ± 0.05	2.52a ± 0.05	1.59a ± 0.06	0.69a ± 0.04	0.99a ± 0.07	1.93a ± 0.07	2.17b ± 0.16
	Nitrophenolates 1×**	4.45a ± 0.13	2.19a ± 0.04	3.22b ± 0.10	1.69a ± 0.05	0.61a ± 0.02	1.02a ± 0.05	3.07b ± 0.19	1.88b ± 0.08
	Nitrophenolates 2×**	–	–	2.35a ± 0.04	1.58a ± 0.02	0.67a ± 0.03	0.89a ± 0.05	2.27a ± 0.13	1.55a ± 0.07
Transpiration rate (μmol H_2O m^{-2} s^{-1})	Control	1.17a ± 0.02	2.97a ± 0.02	2.37b ± 0.03	2.48a ± 0.07	5.35a ± 0.09	6.93a ± 0.19	6.29a ± 0.14	7.68a ± 0.31
	Nitrophenolates 1×	1.51a ± 0.11	3.56a ± 0.21	2.12a ± 0.04	2.15a ± 0.05	6.39b ± 0.09	7.14a ± 0.17	6.08a ± 0.28	7.09a ± 0.21
	Nitrophenolates 2×	–	–	2.51b ± 0.03	2.66a ± 0.07	6.04b ±0.14	7.62a ± 0.25	6.63a ± 0.29	8.03a ± 0.21

		21	28	38	54	68
Growing season 2008						
Stomatal resistance (s cm^{-1})	Control	0.81a ± 0.02	1.22a ± 0.05	0.53a ± 0.04	0.83a ± 0.05	1.71a ± 0.07
	Nitrophenolates 1×	1.25b ± 0.04	1.37a ± 0.05	0.49a ± 0.04	0.91a ± 0.06	3.18b ± 0.23
	Nitrophenolates 2×	1.36b ± 0.07	2.01b ± 0.11	0.51a ± 0.04	0.77a ± 0.04	1.98a ± 0.11
Transpiration (μmol H_2O m^{-2} s^{-1})	Control	4.80b ± 0.07	4.56b ± 0.07	6.77a ± 0.14	8.02a ± 0.23	9.05a ± 0.40
	Nitrophenolates 1×	4.40b ± 0.12	3.98a ± 0.06	8.05b ± 0.16	8.89a ± 0.33	7.09a ± 0.53
	Nitrophenolates 2×	3.99a ± 0.11	3.69a ± 0.10	7.25a ± 0.18	8.74a ± 0.27	8.95a ± 0.72

Presented data are mean ± SE, $n = 24$ (2007) and 36 (2008)

* In 2007 growing season plants were grown in 25-l pots placed in the soil of plots of a particular combination

** 1× and 2× refer to single or double spray respectively

*** Values differing significantly at $\alpha = 0.05$ as determined by LSD of Student's t test

Table 7 Changes in expression of genes involved in lignin biosynthetic and metabolic processes in *A. thaliana* plants treated with nitrophenolates

Gene ID	Gene description	Function	Change level[a]
AT3G02130	Leucine-rich repeat transmembrane protein kinase, putative contains Pfam profile: Eukaryotic protein kinase domain	Lignin metabolism	2.01
AT1G67980	Caffeoyl-CoA 3-O-methyltransferase, putative similar to GI:2960356 [Populusbalsamifera subsp. trichocarpa], GI:684942 [Medicago sativa subsp. sativa]	Lignin biosynthesis	2.56
AT1G76470	Cinnamoyl-CoA reductase family similar to cinnamoyl-CoA reductase GB:CAA56103 [Eucalyptus gunnii], Pinustaeda [GI:17978649]; contains non-consensus GG acceptor splice site at exon 4	Lignin biosynthesis	9.09
AT1G33030	O-methyltransferase family 2 protein similar to caffeic acid 3-O-methyltransferase [SP|Q00763] [Populustremuloides], catechol O-methyltransferase [GI:4808524] [Thalictrumtuberosum]	Lignin biosynthesis	17.56

[a] Compared to plants untreated with nitrophenolates

and higher yield. Nearly optimal conditions in 2008 caused a higher number of laterals. However nitrophenolates acts well in stress conditions; therefore optimal conditions could be a reason why accumulation of biomass and yield were lower in this season.

Nitrophenolate application had an effect on yield at the WULS-SGGW field in Poland as well. In 2007, the yield was 35 % higher than in the control. However, in the 2nd year of the study (2008) the increase was only approximately 4 %. This difference between years can be explained by the fact that plants were under stress caused by a spring frost in 2007. It is generally thought that the positive effect of nitrophenolates is much more evident when the plants are grown under stressful conditions (Gawrońska et al. 2008; Przybysz et al. 2008). Moreover, according to some authors, when plants are grown under near-optimal conditions, the positive effects of nitrophenolates may not be observed at all (Vavrina 1998; Gawrońska et al. 2008; Krawiec 2008).

The results of this study also demonstrated that the increase in yield is a consequence of increased stimulation of generative rather than vegetative development. This result has also been reported by other authors for oilseed rape (Budzyński et al. 2008; Przybysz et al. 2008) and other species (Djanaguiraman et al. 2005a; Kozak et al. 2008). In addition to the above-mentioned increase in yield, nitrophenolate application increased biomass production as well. The greater biomass accumulation was the result of increased efficiency of the photosynthetic apparatus, which was manifested by (1) an increase in the intensity of photosynthesis, (2) a higher maximum photochemical efficiency of PSII, (3) an improved performance index and (4) a higher total chlorophyll content, especially later in the vegetative season, suggesting a delay in senescence.

These results are in line with those of Djanaguiraman et al. (2005a); Gawrońska et al. (2008); Wrochna et al. (2008) and Djanaguiraman et al. (2009), who also reported an increase in biomass accumulation, intensity of photosynthesis and chlorophyll content as well as improved chlorophyll *a* fluorescence parameters due to nitrophenolate application. All nitrophenolate-induced changes were more evident in the year when a spring frost occurred, supporting the aforementioned statements by some authors that the positive effect of nitrophenolates can be more clearly observed when the plants are affected by stress.

The changes in the chemical composition of pods led to the hardening of the pod walls and the mechanical prevention of egg laying. An antioviposition effect could be the other reason for repelling, because pod midge females evaluate the plants as unsuitable for the development of the offspring. The antioviposition and repellent effects of phytochemicals are the most common defence strategies of plants to pests, which has been confirmed in several works (Pavela 2010, 2011).

Assuming that the results of this study are reproducible under various conditions, the application of nitrophenolates holds great promise for lowering chemical input into the environment and increasing plants' ability to cope with environmental stresses while simultaneously enhancing yield. Therefore it can be concluded that nitrophenolates (in the form of Atonik) positively affect oilseed rape plants by reducing pod damage caused by brassica pod midge (*Dasineuranapi*, Loew). The degree of this reduction is similar to that induced by the tested insecticides. A reduction of the pod damage caused by brassica pod midge corresponds with an increase in lignin content in pods, which further coincides with the upregulation of genes related to lignin biosynthesis and metabolism. The nitrophenolate-induced increase in seed yield is associated with

greater stimulation of generative rather than vegetative development and with an increase in biomass production. The nitrophenolate-induced increase in biomass accumulation is attributed to an increase in the efficiency of the photosynthetic apparatus, which is evidenced by an increase in the intensity of photosynthesis and the chlorophyll content as well as higher values of chlorophyll *a* fluorescence parameters. The positive effect of nitrophenolates becomes more profound when plants are under the influence of stress factors, regardless of whether the stress is biotic or abiotic in nature. The application of nitrophenolates holds promise for reducing chemical input into the environment.

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