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Research article

O_3 pollution in a future climate increases the competition between summer rape and wild mustard

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ABSTRACT

The initial aim of this study was to evaluate an effect of elevated CO_2 concentration and air temperature (future climate) and O_3 pollution on mono- and mixed-culture grown summer rape (*Brassica napus* L.) and wild mustard (*Sinapis arvensis* L.). The second task was to reveal the mechanisms of the shift in plants' competitiveness in response to single and combined environmental changes. Plants were grown in mono- and mixed-cultures under current climate (CC) (400 µmol mol^{-1} of CO_2, 21/14 °C day/night temperature) or future climate (FC) conditions (800 µmol mol^{-1} of CO_2, 25/18 °C day/night temperature) with and without O_3 treatment (180 µg m^{-3}). Competition had relatively little effect on growth of both species at current climate, independent of O_3 treatment. In contrast, competitive effect of both plant species considerably increased under FC, and especially FC + O_3 conditions, when growth of mixed-culture rape reduced up to 48% and that of wild mustard up to 80%. The mechanisms of elevated competitiveness of rape under the future climate consisted of better antioxidative protection, particularly elevated total antioxidative capacity and activities of peroxidase and ascorbate peroxidase. Whereas stronger oxidative damage, disproportionally high activities of H_2O_2 scavenging enzymes and lower pool of soluble sugars in mixed-culture wild mustard reduced its competitiveness under FC + O_3 conditions. In conclusion it must be pointed out, that regardless improved competitive abilities of rape under FC and FC + O_3 conditions, competition with wild mustard reduced growth, indicating increased weed-induced yield losses in the future climate, especially with concomitant intensification of O_3 pollution.

1. Introduction

Weeds are an important pest in agriculture. Despite the advanced technological achievements in weed control, crop yields can be reduced by up to 34% due to competition by weeds (Clements et al., 2014). Increased spread and/or aggressiveness of invasive and native weeds (Peters et al., 2014) may further increase this problem in the future. Weeds' characteristics, such as higher genetic diversity and phenotypic plasticity, give them an advantage over many crop species, especially under the changing environmental conditions (Ziska and Runion, 2006; Clements et al., 2014). These opportunistic plants are typically late-emerging thermophile (Peters et al., 2014) or C_3 weeds (Davis and Ainsworth, 2012), that are favoured by warmer climate and/or elevated CO_2. Competitiveness, adaptation, and stress tolerance are the characteristics by which weed species secure their survival in a variety of environmental conditions. Therefore, agriculture may be jeopardized by intensified weed competition as an indirect effect of climate change (Chauhan and Johnson, 2011).

One of the main reasons of climate change is increasing atmospheric CO_2 concentration. It is projected that CO_2 concentration will reach the level of 700 ppm, leading to elevation in air temperature by up to 4 °C at the end of this century (IPCC, 2013). The most important response of plants to elevated CO_2 is an increase in net photosynthetic rate, leading to improved growth and productivity (Clements et al., 2014).

Increasing concentrations of tropospheric ozone (O_3) is another global ecological problem of increasing significance. Since the beginning of the XX century O_3 concentration in Europe has doubled and currently reaches 100 µg m^{-3} (Timonen et al., 2004). The primary negative effect of ozone is oxidative damage, since O_3 produces active oxygen species (AOS), such as O_2^-, H_2O_2, OH^-, immediately after entering the apoplast through stomata (Mills and Harmens, 2011). The initial increase of AOS concentration is followed by the secondary

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oxidative burst, caused by signal transduction responses, impaired photosynthesis and cellular damage (Castagna and Ranieri, 2009). Oxidative stress is universal plants' response to multiple environmental stress agents. AOS can damage the most important biomolecules, such as proteins, lipids and nucleic acids (Gill and Tuteja, 2010; Pinchuk et al., 2012). An optimal level of AOS in different cell compartments is maintained by antioxidative system, which consists of antioxidative enzymes and non-enzymatic antioxidants – ascorbate, glutathione, flavonoids, carotenoids, etc. These antioxidative compounds are responsible for plants' tolerance to many biotic and abiotic stressors (Gill and Tuteja, 2010).

Crop responses to elevated CO_2 and other environmental factors is well investigated. However, our understanding about combined effects of climate change and other concomitant environmental factors is very limited. Increasing evidence suggests that elevated CO_2 mitigates the negative effects of many stressors, such as drought and/or temperature (Farfan-Vignolo and Asard, 2012; AbdElgawad et al., 2015), Zn toxicity and N limitation (Naudts et al., 2014). On the other hand, interaction of elevated CO_2, O_3 and temperature usually results in lower stimulation of crops' growth than elevation of CO_2 alone (Clausen et al., 2011; Clements et al., 2014). Similarly, positive effects of elevated CO_2 might be reduced or even reversed by competition with weeds, whose growth will also be promoted by higher CO_2 concentration (Valerio et al., 2013; Clements et al., 2014). Competition between crops and weeds under increasing ozone concentration, could be determined by differential O_3 tolerance (Fuhrer, 2009). However, competition *per se* may alter plant response to other environmental factors (Ziska, 2001) and thus modify crop competitive abilities in the changing environment. Similarly, Menéndez et al. (2017) have evidenced that competitive interaction has greater role in determining plants' responses to O_3 than species sensitivity to this xenobiotic.

Experimental data of weed-crop competition under combined changes in temperature, CO_2 and other abiotic stress factors are very scarce. Therefore, we investigated the competition between the C_3 crop summer rape (*Brassica napus* L.) with C_3 weed wild mustard (*Sinapis arvensis*) under current and future climate conditions (elevated CO_2 and temperature), with and without elevated O_3 levels. Rape is one of the most important crops, mainly used for seed oils (both edible oil and biofuel) as well as for fodder. The changing climate, however, can adversely affect the rape's harvest (He et al., 2017) and oil quality (Namazkar et al., 2016). Sharp reduction in seed biomass, average content of oil and fatty acids was detected in rapes exposed to FC conditions (elevated CO_2 and temperature), especially acting together with higher O_3 concentrations. These findings demonstrate the need and importance of crop researches embracing combined effects of FC and air pollutants (Namazkar et al., 2016). Apart from this, various pests, weeds and diseases are detrimental for rape crops. The most problematic weeds for these crops are broad leaf *Brassicaceae* weeds. One of the best examples is wild mustard, a prevalent winter annual weed, which is particularly difficult to control in oilseed and cereal grain crops (Huang et al., 2001). Since little is known about the mechanisms, that determine the effects of integrated environmental changes on the interaction between agricultural crops and wild plants, the aim of this study was to examine an effect of future climate and ozone pollution on mono- and mixed-culture grown summer rape and wild mustard and to reveal the biochemical and physiological mechanisms of the shifts in plants' competitiveness in response to single and combined environmental changes. Based on the previous findings we supposed an increased weeds' competitiveness towards the crop species under the future climate (elevated air temperature and CO_2 concentration) and/or O_3 pollution. Secondly, we hypothesized that the mechanisms of the shifts in weed/crop competition include the complex changes in photosynthetic and antioxidative systems, as well as oxidative damage in response to the neighbouring plant species.

2. Materials and methods

2.1. Growing conditions and experimental design

Experiments were conducted in phytotron chambers at Vytautas Magnus University, Lithuania. Summer rape (*Brassica napus* L., variety 'Fenje') and wild mustard (*Sinapis arvensis*) were grown in a mixture of field loam soil, perlite and sand (volume ratio 5:3:2), in 3 l plastic pots. Sixteen plants per pot were grown in monoculture; 8 seedlings of wild mustard were grown in addition to 16 rapes in the mixed-culture conditions.

All plants were germinated at current climate (CC), corresponding 21/14 °C day/night temperature and 400 μmol mol^{-1} of CO_2. The treatments of O_3 and/or future climate (FC) were started at the 10–11th BBCH growth stage (Meier, 2001) and lasted 3 weeks. Future climate conditions corresponded 25/18 °C day/night temperature and 800 μmol mol^{-1} of CO_2. Ozone-exposed plants were treated with 180 μg m^{-3} O_3 concentration, whereas plants without O_3 treatment were growth in charcoal filtered air with ~0 μg m^{-3} O_3 concentration. Consequently, investigated plants were grown in four different sets of environmental conditions: CC and FC with or without O_3 treatment (CC, CC + O_3, FC, FC + O_3) (Table 1). Considering 3 sets of species composition (monoculture rape, monoculture wild mustard, mixed-culture plants), experimental setup consisted of 12 different treatments. All treatments were run in 3 replicates, based on the previous researches (Ziska, 2000; Chauhan and Johnson, 2011; Januškaitienė et al., 2018). In order to minimalize variability of plant response within individual experimental treatments, randomization and regular spatial rearrangement of the pots were applied (Poorter et al., 2012).

The following climatic conditions were uniform in all chambers: photoperiod of 14 h, relative humidity of ~70% and 230 μmol m^{-2} s^{-1} photon flux density of photosynthetically active radiation (PAR), provided by day-light luminescent lamps (Philips, Waterproof OPK Natural Daylight LF80) and high-pressure sodium lamp (Philips MASTER GreenPower CG T 600 W). Complex fertilizers were applied to all pots at the sowing day (NPK 12-11-18 + microelements, 90 kg N ha^{-1}) and at the beginning of the treatments (level of nitrogen increased until 150 kg N ha^{-1}). Climatic conditions in the growth chambers were set and controlled automatically. The concentration of CO_2 was manipulated by controlling the amount of injected CO_2 gas and the chamber conditioner. The O_3 gas was supplied by ozone generator OSR-8 (Ozone solutions, Inc) and the concentration was automatically monitored and regulated by ozone sensors OMC-1108. The climate program was controlled by the IGSS 9-13175 software.

2.2. Determination of plants growth

At the end of O_3 and/or FC treatments 5 plants from each pot were harvested and divided into shoots and roots for determination of dry

Table 1
The experimental setup and conditions of different treatments applied for monoculture rape and wild mustard seedlings as well as for mixed-culture plants.

	Current climate (CC) 21/14 °C day/night temp., CO_2 400 μmol mol^{-1}	Future climate (FC) 21/14 °C day/night temp., CO_2 400 μmol mol^{-1}
O_3 control 0 μg m^{-3}	CC	FC
+ O_3 180 μg m^{-3}	CC + O_3	FC + O_3

biomass. Roots were carefully washed, and the samples were dried at 70 °C until a constant dry weight was obtained (~5 days). Biomasses of roots and shoots samples were recalculated into dry biomass per plant and root/shoot ratio. Four plants from each pot were used for evaluation of leaf area. Leaves were removed and scanned using CanoScan 4400F scanner (Canon, USA). Leaf area was evaluated using GIMP 2.8 software.

2.3. Gas exchange measurements

Gas exchange was measured using a portable closed infra-red gas analyser LI-COR 6400 (LI-COR, Inc., Lincoln, NE, USA) with randomly selected the youngest fully expanded leaves at the last day of the treatment between 10:00 a.m. and 5:00 p.m. Photosynthetic rate (*A*, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), transpiration rate (*E*, mmol H_2O m^{-2} s^{-1}) and intracellular CO_2 concentration (C_i, μmol CO_2 mol air^{-1}) were recorded automatically for approximately 10 min, at 5 s interval. Air flow rate through the assimilation chamber was set at 500 μmol s^{-1}, PAR at the leaf surface was of ~205 μmol m^{-2} s^{-1}. After embedding in the assimilation chamber, leaves were allowed to acclimate for ~5 min, until gas exchange was in a steady state. Water use efficiency (WUE, μmol CO_2 mmol H_2O^{-1}) was calculated as CO_2 assimilation rate (*A*) dividing by transpiration rate (*E*).

2.4. Determination of biochemical parameters

Leaves of plants were harvested and immediately frozen with liquid nitrogen. Frozen and grounded biomass was stored at 80 °C until the analyses. 50-100 mg of leaf tissue was weighted into the 2 ml Eppendorf test-tubes and pulverized in liquid nitrogen with MagNALyser Instrument (Roche, Vilvoorde, Belgium). The powder of the tissue was homogenized in 1 ml 80% (v/v) ethanol, the supernatant was used for biochemical analysis (except antioxidative enzymes). All measurements were run in 3 biological and 4 analytical replicates.

The concentration of TSS was determined using Anthrone reagent (Leyva et al., 2008). Tissue extract was transferred into microplate and mixed with 0.1% (w/v) Anthrone, diluted in concentrated sulfuric acid. Reaction mixture was incubated at 85 °C for 45 min. After incubation, the absorbance of the reaction mixture was measured at 620 nm with Microplate reader (Synergy Mx, BiotekInstruments Inc., Vermont, USA), using glucose as a standard.

The concentrations of chlorophylls a + b and carotenoids in tissue extract were calculated by equations: $C(chl\ a + b) = 5134 \times OD_{664} + 20 \times OD_{648}$, $C(carot) = 4695 \times OD_{470} - 0{,}268 \times C(chl\ a + b)$, where C(chl a + b) – concentration of chlorophylls a + b (μg/ml), C(carot) – concentration of carotenoids (μg/ml), OD_{664}, OD_{648} and OD_{470} – absorption of leaf tissue extract at appropriate wavelength. The concentrations of photosynthetic pigments in plant leaves (mg g^{-1} FW) were calculated evaluating tissue biomass and the amount of ethanol used for extraction.

Lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA) using a thiobarbituric acid-malondialdehyde (TBA-MDA) assay (Murshed et al., 2008). The supernatant of the tissue extract was transferred into microplate and mixed with 0.5% (w/v) TBA, diluted in 20% (w/v) trichloroacetic acid. The reaction mixture was incubated at 85 °C for 45 min. The reaction was terminated by transferring the microplate into ice bath. The absorbance was measured at 440, 532 and 600. The concentration of MDA in tissue extract was calculated by equation: $(6.45 \times (OD_{532} - OD_{600}) - 0.56 \times OD_{440})/0.478$, where OD_{532}, OD_{600} and OD_{440} – absorption of reaction mixture at appropriate wavelength. MDA concentration if plant leaves were calculated evaluating tissue biomass and all dilutions throughout analysis.

Total antioxidant capacity (TAC) of leaf extracts was estimated by means of the Ferric Reducing Ability of Plasma (FRAP) assay (Benzie and Strain, 1996). The tissue extract was mixed in microplate with FRAP reagent. The reagent contains 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-Tris (2-pyridyl) s-triazine (TPTZ) prepared in 40 mM HCl and 20 mM $FeCl_3$. The absorbance was measured at 600 nm 6-Hydroxy-2,5,7,8-tetra methylchroman-2-carboxylic acid (Trolox) was used as a standard.

Polyphenols' concentration was determined by using Folin–Ciocalteu reagent (Zhang et al., 2006). Tissue extract was mixed with 10 times diluted Folin–Ciocalteu reagent and saturated Na_2CO_3. After 1 h incubation in the dark the absorbance was measured at 725 nm. Gallic acid was used as a standard.

For determination of flavonoids' concentration, the leaf tissue extract was mixed with 10% (w/v) aluminium chloride and 1 M potassium acetate. The absorbance was measured at 415 nm after 30 min incubation at room temperature (Chang et al., 2002). Quercetin was used as a standard.

Activities of the following antioxidative enzymes were detected: catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX) and glutathione reductase (GR). Frozen tissues (0.05 g) were homogenized in 1 ml 0,05 M K-phosphate buffer (pH 7.0). Extracts were homogenized by MagNALyser (20 s, 6000 rpm), extraction buffer contained 2% (w/v) PVPP, 0.4 mM EDTA, 0.2 mM PMSF. Activities of antioxidative enzymes were measured spectrophotometrically in microplates. Activities of DHAR and MDHAR and GR were determined as was described by Murshed et al. (2008). Catalase activity was assayed by monitoring consumption of H_2O_2 at 240 nm. Peroxidase (POX) activity was measured according to Kumar and Khan (1982) by monitoring the production of purpurogallin at 430 nm.

2.5. Statistical analysis

Fisher's Least Significant Difference (LSD) test was applied to evaluate statistically significant differences between the means of investigated parameters. The threshold for significance was a p-value < 0.05. Factorial ANOVA was used to evaluate the effects of competition, O_3 and FC treatments and their combinations on crop's and weed's growth as well as on photosynthetic and biochemical parameters. All analyses were performed by STATISTICA 8 software.

3. Results

3.1. Plant growth

Future climate positively affected rape's growth: 83% and 38% increases were detected for shoot biomass and leaf area, respectively (Fig. 1A,D); lower but significant increase was also noticed for root biomass (Fig. 1B). Therefore rape's root/shoot ratio reduced under FC conditions (Fig. 1C) Wild mustard was less tolerant than rape to ozone at CC. Biomass of weed's shoot and root reduced by 31% and 52% ($p < 0.05$) under O_3 treatment, respectively (Fig. 1E and F). FC had negligible effect on weed growth, however, it markedly reversed the negative O_3 impact: monoculture grown weeds had significantly higher biomass at FC + O_3 compared to CC conditions (Fig. 1E and F).

Weed had negligible effect on the growth of rape at current climate, independent of O_3 pollution. Only root biomass was slightly reduced at CC conditions (Fig. 1B). Competition-induced growth inhibition sharply increased at FC and especially at FC + O_3 conditions. The most drastic changes were found for leaf area, which reduced by 35% and 48% at FC and FC + O_3 conditions ($p < 0.05$) (Fig. 1D). Consistent with these results, ANOVA analysis showed that FC and weed competition significantly affected rape's growth (A1). Growth of wild mustard at CC was stronger affected by competition than rape: root biomass and leaf area significantly reduced in mixed-culture grown weeds (Fig. 1F,H). Similarly to rape, the negative competition effect on wild mustard also

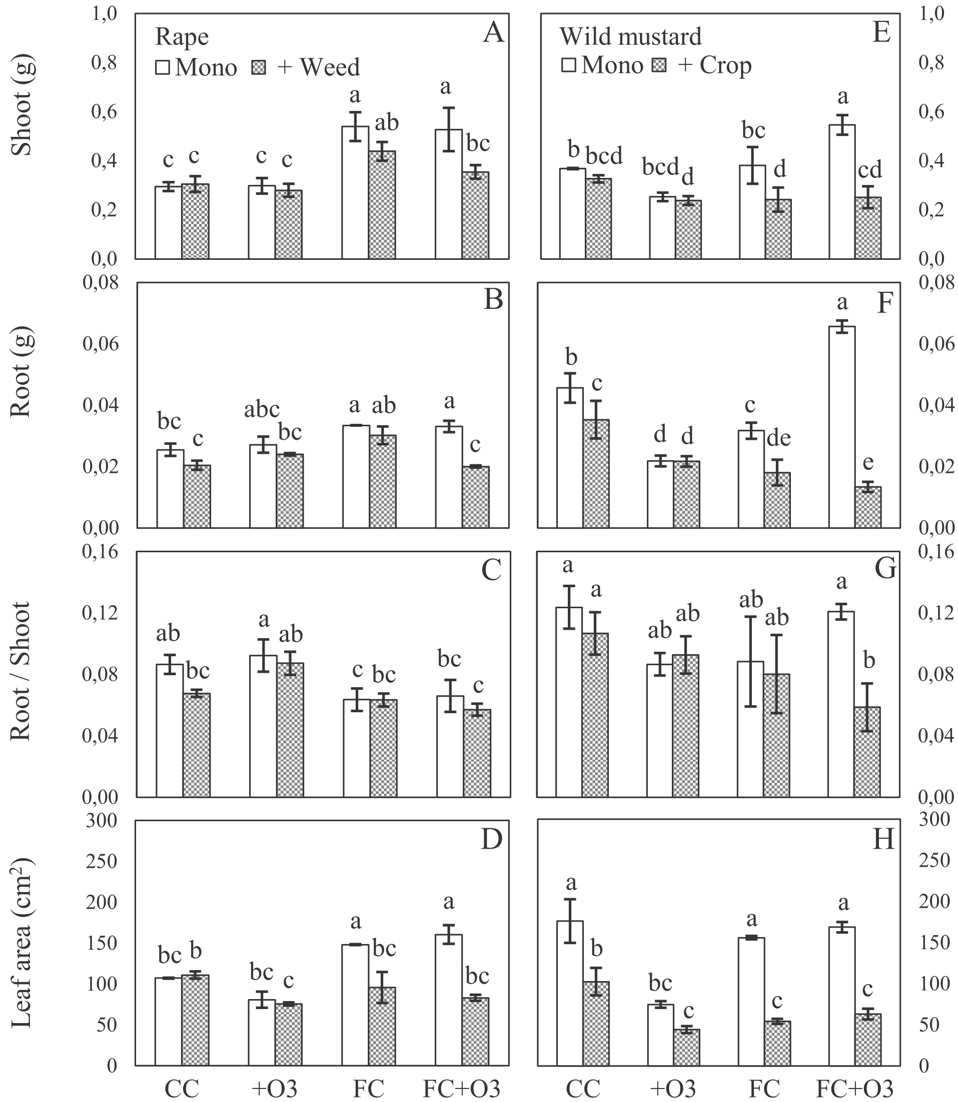

Fig. 1. Dry biomass (DW) of root and shoot, root/shoot ratio and leaf area of summer rape and wild mustard grown under different climate conditions (CC – current climate, +O3 – O_3 treatment at current climate, FC – future climate, FC + O3 – O_3 treatment at future climate). The whiskers are standard errors (n = 3); different letters above the columns indicate statistical significant differences between the means.

sharply increased at FC and especially at FC + O_3 conditions, when root growth was suppressed by 80% and root/shoot ratio reduced by ~2 times (Fig. 1F and G). It must be pointed out, that competition-induced crop's growth inhibition was considerably lower: rape's root growth was suppressed by only 40% (Fig. 1B), shoot biomass and LA of rape was also less affected by competition than that of wild mustard. This finding indicates reduced wild mustard competitiveness and contradicts our hypothesis that competitiveness of weed increases at future climate (FC and/or FC + O_3 conditions).

3.2. Photosynthesis and gas exchange parameters

To investigate the physiological basis of the differential response of the two species to FC and O_3, we first analysed gas exchange parameters. Ozone negatively affected photosynthetic rate (A) in both plant species, whereas stimulating FC tendency was insignificant (Fig. 2A,E).

Rape's A tended to decrease due to competition with wild mustard; however, significant weed-induced down-regulation was observed only under CC + O_3 (51%, $p < 0.05$) and FC + O_3 treatment (23%, $p < 0.05$) (Fig. 2A). According to ANOVA, competition effect on A was low, as compared to the effect of O_3 ($F = 47.8$, $p < 0.001$) and climate ($F = 29.7$, $p < 0.001$) (A1). As was expected, stomatal conductance (g_s) and transpiration rate (E) tended to decrease due to O_3 treatment and to increase at FC in both plant species. G_s and E in rape were not altered by weed competition at CC; whereas down regulation of these parameters was detected under CC + O_3 and FC conditions (Fig. 2B and C). WUE was little affected by competition with wild mustard, significant reduction was detected only at CC conditions (Fig. 2D).

3.3. Photosynthetic pigments

To investigate if differences in photosynthesis were entirely related to stomatal conductance we also measured the levels of chlorophyll and carotenoid pigments. FC and elevated O_3 tended to increase the concentrations of chlorophylls and carotenoids in monoculture rape's leaves (Fig. 3A and B), corresponding to slight A stimulation (Fig. 2A). In contrast, reduction was observed in wild mustard in response to O_3 treatment (Fig. 3D and E). This effect was even more intensified by competition with rape at CC + O_3 conditions.

Competition-induced changes in the pool of photosynthetic pigments were inconsistent and relatively low in both plant species. Strong and significant reduction in concentration of chlorophylls and

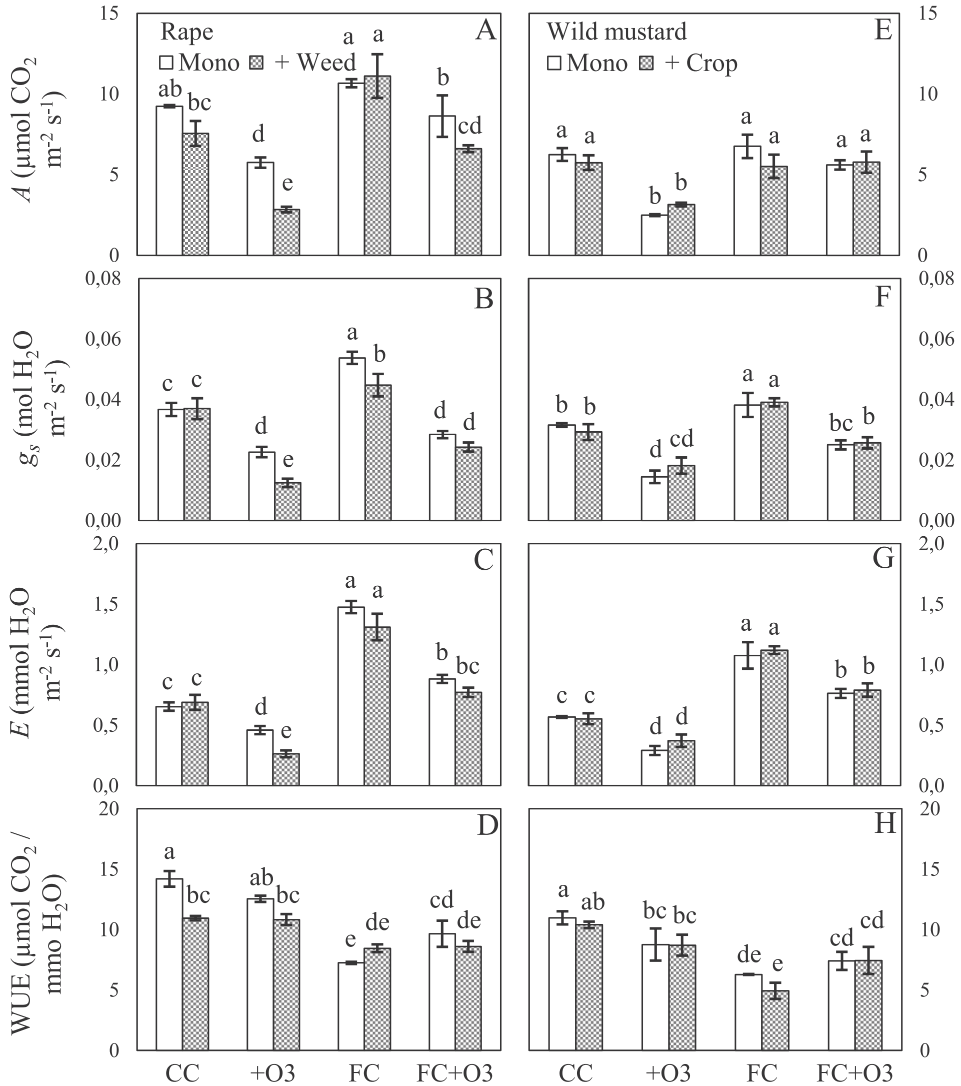

Fig. 2. Photosynthetic parameters of summer rape and wild mustard grown under different climate conditions. Designations as in Fig. 1.

carotenoids in mixed-culture compared to monoculture rape (Fig. 3A and B) and wild mustard (Fig. 3D and E) was noticed only at CC + O_3 conditions. In the case of rape, this reduction was closely correlating with reduced photosynthetic rate due to competition under O_3 treatment (Fig. 2A).

3.4. Accumulation of soluble sugars

Downstream of photosynthesis, the level of total soluble sugars (TSS) sharply increased (more than 2-fold) in monoculture crop at FC compared to CC conditions (Fig. 3C). Even higher increase (about 3-fold) was detected in monoculture weed under FC and FC + O_3 conditions (Fig. 3F). Climate was the main factor of variation of TSS in rape ($F = 38$, $p < 0.0001$) and wild mustard ($F = 72.1$, $p < 0.0001$) (A1).

Considering weed competitive effect on accumulation of TSS, its concentration significantly increased only in mixed-culture rape compared to monoculture ones (82%, $p < 0.05$) under O_3 treatment at current climate (Fig. 3C). Whereas strong and significant reduction (38%, $p < 0.05$) was characteristic for mixed-culture compared to monoculture weed at FC + O_3 conditions (Fig. 3F). Thus, FC increased TSS in absence of a clear induction of photosynthetic rate, and the presence of weeds under CC + O_3 treatment also strongly increased TSS, which is opposite to the effects on photosynthesis and does not correlate with the absence of an effect on growth.

3.5. Lipid peroxidation and antioxidative capacity

Because the main consequence of elevated O_3 is thought to be the induction of oxidative stress, we investigated oxidative damage caused by the treatments and the response of the plants' antioxidant regulation system. As expected, ozone significantly increased oxidative damage in rape's leaves under current and future climate (Fig. 4A). In contrast, lipid peroxidation tended to reduce in monoculture wild mustard, exposed to CC + O_3, FC and FC + O_3 conditions (Fig. 4E). Total antioxidative capacity (TAC) in leaves of monoculture rape and wild mustard sharply increased (2.5 times) at FC and FC + O_3 conditions (Fig. 4B,F). Consistent with these results, future climate was the main factor for variation of TAC in leaves of rape ($F = 776.8$, $p < 0.0001$) and wild mustard ($F = 769.2$, $p < 0.0001$) (A2). However, the induction of TAC by FC and FC + O_3, was at best sufficient to limit, but not to prevent the induction of oxidative damage.

The impact of interspecific competition on lipid peroxidation in both plant species varied between negligible effect at CC, significant reduction at CC + O_3 and considerable increase (39% and 20%, $p < 0.05$, in rape and wild mustard, respectively) at FC conditions. However, oxidative damage was reduced in rape (16.5%) and increased in wild mustard (20.4%) under FC + O_3 conditions by the presence of the other species (Fig. 4A,E). Competition-induced changes of TAC corresponded to the reduction of oxidative stress under CC + O_3 and

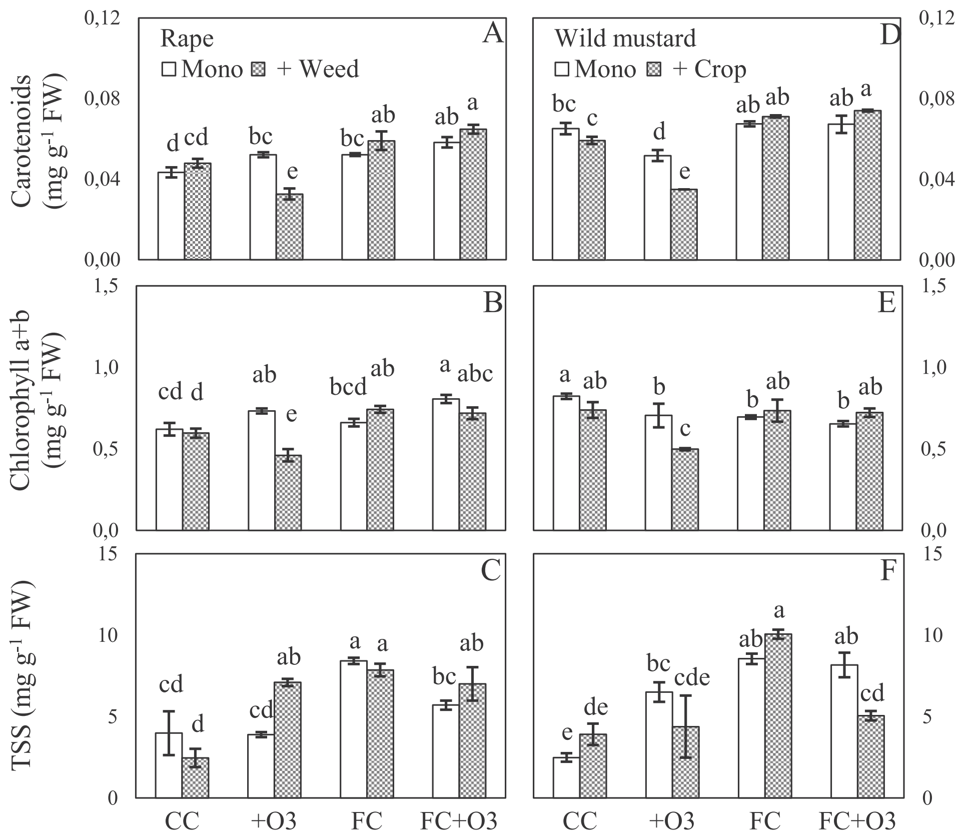

Fig. 3. Concentrations of photosynthetic pigments and total soluble sugars in the leaves of summer rape and wild mustard grown under different conditions. Designations as in Fig. 1.

intensification of it under FC conditions. However, statistically significant increase of TAC was detected in mixed-culture rape under FC, and reduction in mixed-culture wild mustard under CC + O_3 conditions (Fig. 4B,F).

3.6. Polyphenols and flavonoids

To further understand the variations in TAC, we measured the levels of key antioxidant metabolites. The concentration of polyphenols reduced in monoculture plants at FC as compared to CC conditions; in contrast, O_3 tended to increase the pool of phenolic compounds (Fig. 4C,G). The concentration of flavonoids to some extent repeated the variation of polyphenols (Fig. 4D,H). Based on ANOVA analysis, polyphenols and flavonoids were mostly affected by climate conditions in both plant species (A2).

Competition induced significant reduction and increase of polyphenols concentration in plants' leaves at CC + O_3 and FC conditions, respectively (Fig. 4C,G). The changes of flavonoids in plants' leaves followed the similar pattern as polyphenols (Fig. 4D,H). However, exceptionally strong increase (3.6 times, $p < 0.05$) was detected in flavonoids pool in mixed-culture compared to monoculture rape and wild mustard at FC + O_3 conditions (Fig. 4D,H).

Thus, stimulation of TAC by FC (Fig. 4B,F) was not related to these antioxidant metabolites, whereas the increase in rape's TAC by competition under FC conditions could be at least partly determined by higher concentrations of polyphenols.

3.7. Antioxidative enzymes

A second line of defence against oxidative damage involves the activity of antioxidative enzymes. Future climate stimulated activities of H_2O_2 scavenging enzymes in monoculture-grown rape. Activities of CAT (Fig. 5A) and GPX (Fig. 5D) increased by 2.7 and 3.9 times ($p < 0,05$), respectively; instead, POX (Fig. 5B) activity was elevated by FC + O_3 treatment. Activity of DHAR, responsible for recovery of reduced ascorbate, also increased (Fig. 6B); whereas MDAR (Fig. 6C) and GR (Fig. 5C) were downregulated by FC, independent of O_3 treatment. In contrast to rape, antioxidative enzymes in wild mustard were not stimulated by FC; only GPX activity was sharply increased (almost 3-fold) by O_3 treatment (Fig. 5H).

Interspecific competition affected both plant species in similar way, especially under the current climate: MDAR (Fig. 6C,F) and GR (Fig. 5C,G) activities were reduced, whereas GPX (Fig. 5D,H) sharply increased by competition in rape (6.7 times) and wild mustard (2.8 times) ($p < 0.05$). A powerful increase of POX (Fig. 5B,F), APX (Fig. 6A,D) and GPX (Fig. 5D,H) activities was detected in mixed-culture rape and wild mustard as compared to monoculture ones under CC + O_3, FC and/or FC + O_3 conditions. The highest stimulation of these enzymes was observed in mixed-culture wild mustard exposed to FC + O_3 conditions, when activity of these H_2O_2 scavenging enzymes increased from 10.8 to 12.6 (POX and APX, respectively) (Figs. 5F and 6D) to 20.7–207 times (GPX) (Fig. 5H).

4. Discussion

In order to elucidate the shift of competitive abilities of summer rape with C3 weed wild mustard in the changing environment (climate change and O_3 pollution), we examined O_3 effects on monoculture-grown plants and on weed-crop interaction at current climate and future climate on mono- and mixed-culture-grown plants with and without ozone treatment.

4.1. Response of monoculture plants to O_3 pollution at current climate

Ozone is strong atmospheric oxidant, penetrating plasmalemma through stomata and forming various AOS after reaction with water

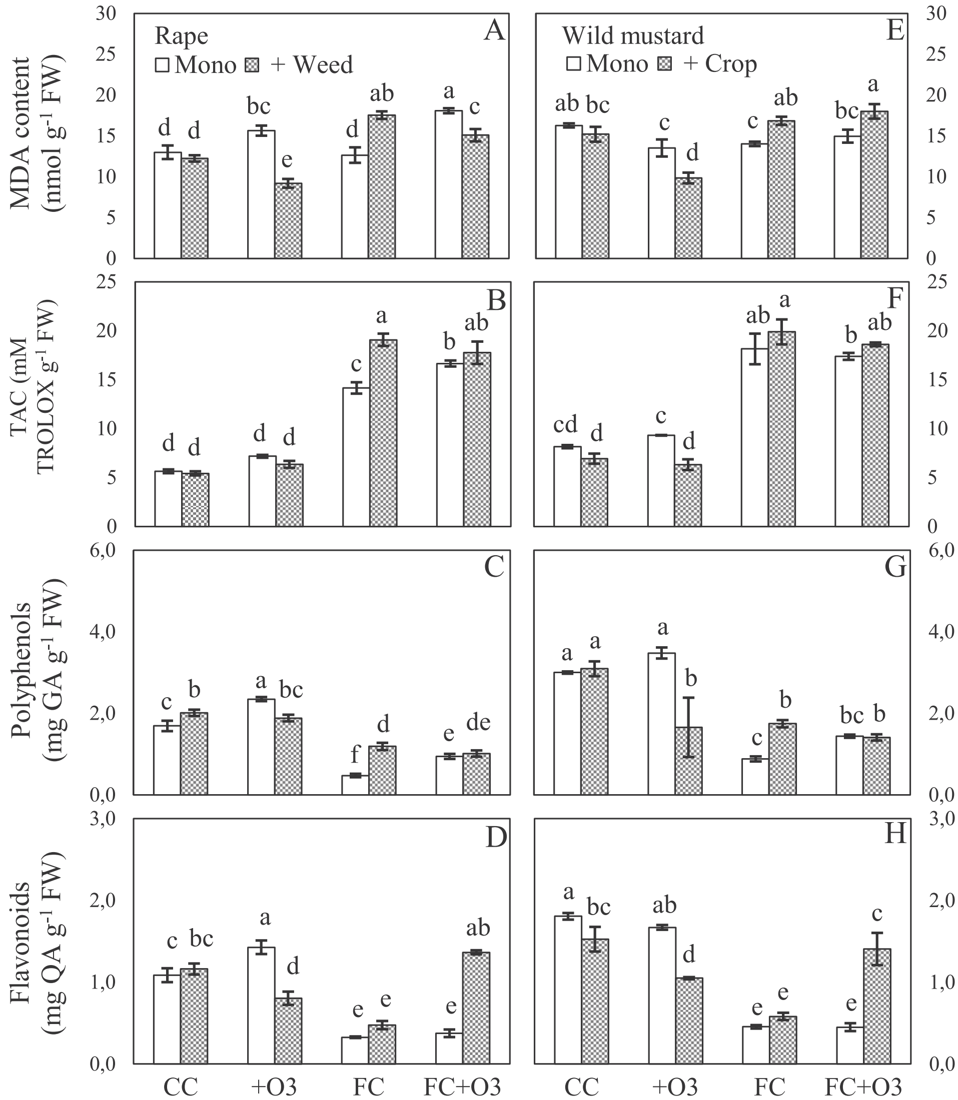

Fig. 4. The level of membrane oxidative damage (MDA concentration), total antioxidant capacity (TAC) and concentrations of polyphenols and flavonoids in the leaves of summer rape and wild mustard grown under different conditions. Designations as in Fig. 1.

(Castagna and Ranieri, 2009). Our research has shown relatively strong O_3-induced down-regulation of photosynthetic rate and intensification of oxidative stress in monoculture rape; on the contrary, O_3 effect on rape's growth was low and insignificant. Reduction in stomatal conductance, increased concentrations of chlorophylls and low-molecular weight antioxidants (carotenoids, polyphenols and flavonoids) could be responsible for effective prevention of more severe damage. This finding is in accordance with earlier studies, demonstrating negligible O_3 effect on growth and productivity of oilseed rape and barley (Clausen et al., 2011), as well as low growth inhibition vs. strong oxidative damage in O_3-treated barley seedlings (Kacienė et al., 2015).

Oilseed rape is attributed to the moderately ozone-sensitive crops (Fuhrer, 2009; Mills and Harmens, 2011), whereas almost nothing is known about weed wild mustard sensitivity to O_3. In the current study wild mustard was found to be less tolerant than rape to ozone treatment, which induced strong reduction of root and shoot biomass and leaf area as well as degradation of photosynthetic pigments. However, shoot biomass was reduced much weaker than LA, implying biomass allocation from leaves to stems and/or increased thickness of leaves. Increased concentrations of total soluble sugars (TSS) and polyphenols, as well as reduction in lipid peroxidation and stimulation of H_2O_2 scavenging GPX was also detected in O_3-exposed wild mustard, indicating a trade-off between O_3-induced damage and activation of the mechanisms of defence.

4.2. O_3 effects on weed-crop competition under current climate conditions

The presence of wild mustard had a small effect on the growth of the rape at current climate, ozone-free conditions. Growth of wild mustard suffered more than rape from interspecific competition, as weed's root DW and especially LA were significantly reduced. Since relative leaf area at early growth stage is important determinant of shading and weed-induced yield loss at maturity (Kropff and Spitters, 1991; Valerio et al., 2013), our data suggest that competitive impact of wild mustard on rape is relatively low at current climate, at least under investigated plant densities.

Although wild mustard was more sensitive to ozone than rape, its competitiveness with respect to growth did not change considerably under O_3 treatment. This finding contradicts the previous suggestions, that differential O_3 tolerance could determine an outcome of weed-crop competition (Fuhrer, 2009; Li et al., 2013). Regardless negligible changes of growth, a shift was detected at physiological and biochemical level of mixed-culture as compared to monoculture plants. Competition under elevated O_3 concentration induced significant mitigation of oxidative damage, as well as considerable reduction in concentrations of chlorophylls and low molecular weight antioxidants

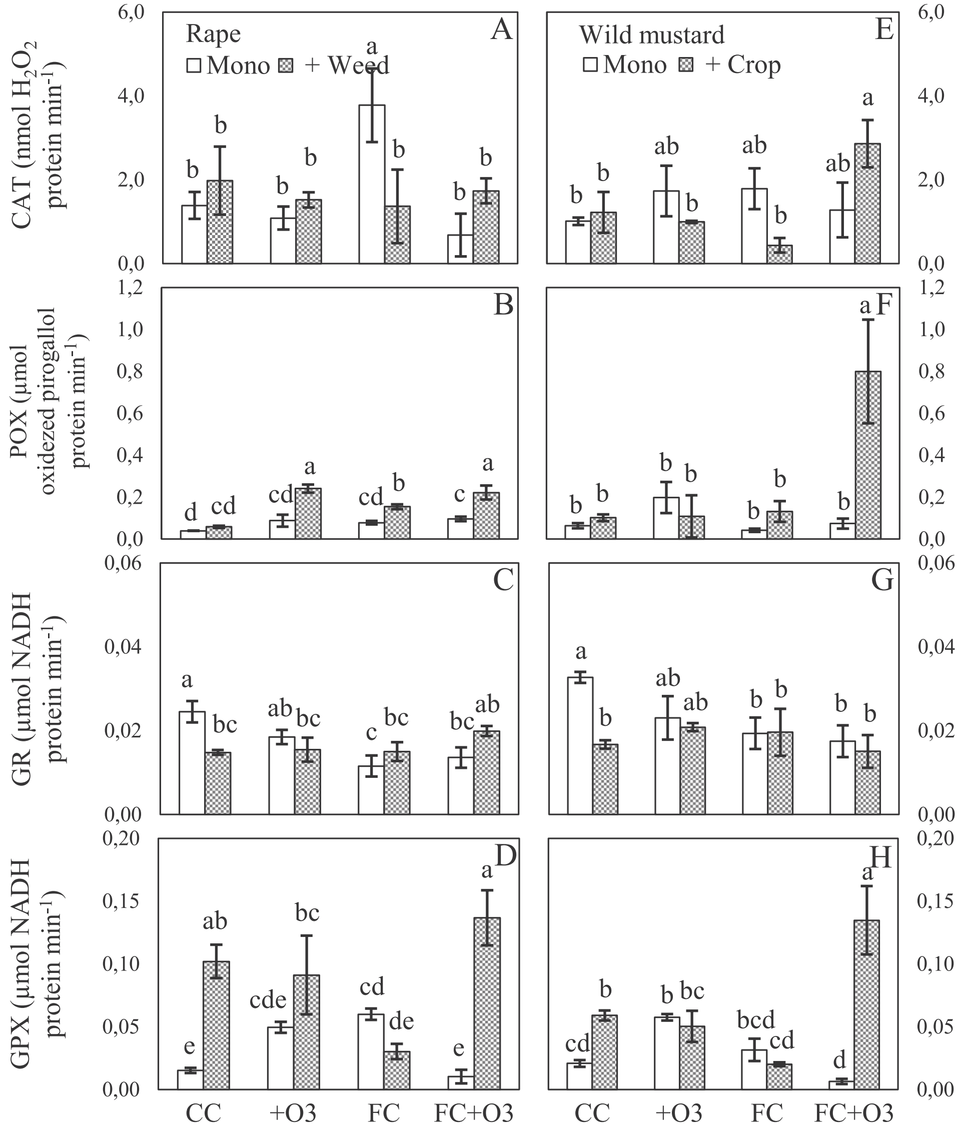

Fig. 5. Activities of catalase (CAT), peroxidase (POX), glutathione reductase (GR) and glutathione peroxidase (GPX) in the leaves of summer rape and wild mustard grown under different conditions. Designations as in Fig. 1.

(carotenoids, polyphenols and flavonoids) in both plant species. Whereas competition-induced changes in antioxidative enzymes and photosynthetic system were significant only for rape: APX and POX activities was elevated, and photosynthetic parameters and WUE reduced in response to weed competition. Reduction in A may be caused by degradation of chlorophyll and impaired activity of mesophyll cells due to combined effect of O_3 and weed interaction, as was already noticed by Li et al. (2016). Apart from this, downregulation of A might be also induced by sharply increased accumulation of TSS instead of direct negative effect of wild mustard. These variations in plants' physiological functions, oxidative damage and the pools of important biomolecules indicate physiological and biochemical response to complex action of O_3 and interspecific competition, though not strong enough to result in significant suppression of growth. However, it can also signify stronger weed-induced damage in the subsequent growth stages of crops exposed to O_3 pollution at the current climate, as was already evidenced by Li et al. (2013).

4.3. Future climate effects on monoculture plants

Positive effect of FC conditions on crops' growth and productivity is widely elucidated in scientific literature (Singh and Agrawal, 2015; Yoon et al., 2009; Aranjuelo et al., 2011; AbdElgawad et al., 2015; Kacienė et al., 2017). Our results also revealed that elevated CO_2 and temperature stimulate rape's growth: biomass of root and shoot, leaf area as well as accumulation of soluble sugars significantly increased. In contrast to considerable growth stimulation, rape's photosynthetic carbon assimilation had risen by much lesser extent. Together with markedly increased transpiration rate, this led to reduced WUE. Among the major obstacles to elevated CO_2-induced photosynthetic stimulation could be strong accumulation of TSS in rape's leaves, as was evidenced in previous researches (Aranjuelo et al., 2011; Kacienė et al., 2017).

Apart from photosynthetic performance, there are other internal factors that contribute to future climate-induced growth stimulation. One of the most important is improved antioxidative system (Singh and Agrawal, 2015; Xu et al., 2015). In agreement with earlier suggestions, a powerful increase in TAC as well as activities of H_2O_2 scavenging antioxidative enzymes (CAT and GPX) and ascorbate recovering DHAR was detected in FC-grown rape. Total antioxidative capacity in plants is maintained by low-molecular weight antioxidants (Naudts et al., 2014), such as carotenoids, which concentration was also elevated in FC–, compared to CC-grown rape. CAT is one of the most efficient antioxidative enzyme, whereas GPX and DHAR are important enzymes that work together in ascorbate-glutathione cycle in many compartments of

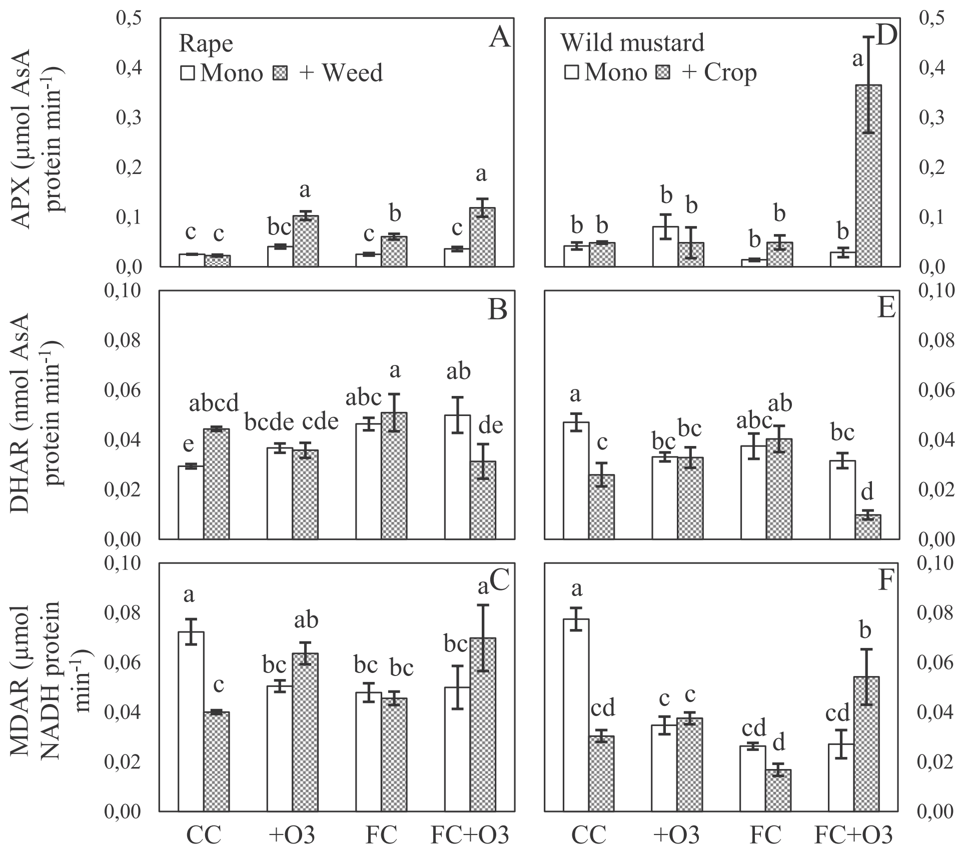

Fig. 6. Activities of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDAR) in the leaves of rape and wild mustard grown under different conditions. Designations as in Fig. 1.

the cell (Mittler et al., 2004). Therefore, activation of these antioxidants reveals the role of both enzymatic and non-enzymatic antioxidative system in FC-induced rape's growth stimulation.

Future climate fractionally modified O_3 effects on rape, whereas completely reversed O_3-induced growth inhibition of wild mustard. The protective role of FC might be explained by mitigated damage on photosynthetic carbon assimilation and improved total antioxidative capacity, supporting the previous findings about the importance of antioxidative system in FC-induced protection against environmental stress factors (Farfan-Vignolo and Asard, 2012; Naudts et al., 2014; AbdElgawad et al., 2015; Xu et al., 2015).

4.4. Combined future climate and O_3 effects on weed-crop competition

Future climate significantly increased the competitive impact of both investigated plant species. Several examples in scientific literature have also demonstrated the intensification of C_3 weed competitive impact on C_3 crops under the climate change (Ziska, 2000; Valerio et al., 2013). Surprisingly, in contrast to our hypothesis, a much greater increase in competitiveness was observed in rape than in wild mustard. This was the most obvious for growth of belowground biomass: the root biomass of wild mustard was suppressed twice as much as the root biomass of rape by interspecific competition. The shift in biomass partitioning, especially with respect to increased root production, is important CO_2-induced effect on plants, ameliorating nutrients and water availability (Runion et al., 2008). Previous studies suggest that increased biomass allocation to roots at future climate could help plants to resist drought stress (Naudts et al., 2014) and competition (Miri et al., 2012). Therefore, sharp reduction of roots growth of wild mustard in response to competition at FC and especially at FC + O_3 conditions could lead to overall reduction in competitiveness of this weed.

The mechanisms which underlie the shift of outcomes of weed-crop competition under the future climate can be explained by the changes at physiological and biochemical level. Some previous studies suggest that photosynthetic performance is important determinant of competition effects at current (Ratnayaka et al., 2003) and future climate (Li et al., 2016; Januškaitienė et al., 2018). However, the differences in photosynthetic rate, WUE and other gas exchange parameters between monoculture- and mixed-culture-grown rape as well as wild mustard were low and mostly statistically insignificant. In contrast, lipid peroxidation and concentration of polyphenols significantly increased in mixed-culture plants under FC conditions, indicating stronger oxidative damage and antioxidative response to interspecific competition. However, TAC and activities of APX and POX were significantly higher only in rape. Therefore, better antioxidative protection seems to be responsible not only for FC-induced growth stimulation of monoculture rape, as was discussed previously, but also for improved rape's competitive abilities under the future climate.

Considering the combined effect of FC and O_3 on weed-crop competition, the most powerful changes were observed in redox parameters (lipid peroxidation, H_2O_2 scavenging enzymes and flavonoids) and TSS. First, activities of POX, APX and GPX sharply increased in mixed-culture, compared to monoculture plants at FC + O_3 conditions. On the one hand, this can lead to better antioxidative protection and lower oxidative damage, as was detected in rape. Overproduction of antioxidants, however, might be ineffective and misleading use of resources, as was a case of mixed-culture wild mustard, which demonstrated increased lipid peroxidation and extremely high activities of POX, APX and GPX. Well-balanced content of antioxidants and mitigation of oxidative damage under the future climate has been already proved to play an important role in *Echinochloa crus-galli* L. competitiveness (Januškaitienė et al., 2018) and grasses resistance (Naudts et al., 2014). Therefore, increased oxidative damage and disproportionally high activities of H_2O_2 scavenging enzymes is one of the reliable mechanisms of reduced wild mustard competitiveness at FC + O_3 conditions.

The second potential reason of the shift in rape and wild mustard competitiveness at FC + O_3 conditions can be considerable reduction of TSS content in mixed-culture wild mustard. Soluble sugars are involved in gene regulation, antioxidative defence and signal transduction (Keunen et al., 2013). Naudts et al. (2014) have found that changes in sugar metabolism, with respect to increased C allocation to non-structural soluble carbohydrates, is one of the major stress-coping strategies of grassland species under the future climate. The results of our study imply that accumulation of TSS also contributes to plant competitiveness in weed/crop interactions. Reduced TSS content in leaves of mixed-culture wild mustard could also influence intense inhibition of root growth and reduction in weed competitiveness via reduced C transport in the phloem.

Sharply increased concentration of flavonoids could be the third mechanism, explaining the severe intensification of plants' competitive impact at FC + O_3 conditions. Flavonoids play a number of functions in plants, such as antioxidative defence, regulation of shoot and root development and allelopathic protection against weeds and pests (Weston and Mathesius, 2013). Almost 4-fold increase of flavonoid content in the leaves of mixed-culture rape and wild mustard allows us to assume a stimulation of flavonoid transport to the roots and soil and hereby increased allelopathic interaction and growth inhibition of both mixed-culture plants. However, plants' allelopathic interaction was not included in the scope of this study; further research is needed to validate the presumption that allelopathic interaction between Brassicaceae weeds and crops might be considerably intensified by concomitant increase in CO_2, temperature and O_3 concentration.

5. Conclusions

The results of this study show that FC conditions stimulate rape's growth via an increase in total antioxidative capacity as well as activities of H_2O_2 scavenging antioxidative enzymes and ascorbate recovering DHAR. Rape and wild mustard interaction effect on plants' growth at current climate is negligible; however, competitive effect of both plant species considerably increases at FC and especially FC + O_3 conditions, when growth of rape was significantly reduced by weed competition. Nevertheless, the competitiveness of rape increases more than that of the wild mustard, negating our hypothesis of increased weeds' competitiveness towards the crop species under the future climate and/or O_3 pollution. The mechanisms which underlie higher rape's competitiveness under the future climate consists of better antioxidative protection, evidenced by elevated TAC and activities of APX and POX. Whereas stronger oxidative damage, disproportionally high activities of H_2O_2 scavenging enzymes (POX, APX and GPX) and considerably decreased pool of TSS in mixed-culture wild mustard determines its sharply reduced competitiveness under FC + O_3 conditions. It must be emphasized, however, that regardless improved competitive abilities of rape, competition with weeds strongly interferes with the crop's growth, indicating increased yield losses due to the presence of weeds in the future climate, especially with concomitant intensification of O_3 pollution. The potential mechanism of growth reduction, which should be deeper investigated in the future, is flavonoid-based allelopathic interaction between rape and wild mustard under FC + O_3 conditions.

CRediT authorship contribution statement

Giedrė Kacienė: Writing - original draft, Writing – review & editing. **Diana Miškelytė:** Data curation, Formal analysis. **Hamada AbdElgawad:** Formal analysis, Writing - original draft. **Gerrit Beemster:** Methodology, Resources, Writing - original draft. **Han Asard:** Methodology, Resources, Writing - original draft, Supervision. **Austra Dikšaitytė:** Data curation, Formal analysis, Visualization. **Jūratė Žaltauskaitė:** Data curation, Formal analysis, Investigation. **Gintarė Sujetovienė:** Data curation, Formal analysis, Investigation. **Irena Januškaitienė:** Data curation, Formal analysis, Investigation. **Romualdas Juknys:** Conceptualization, Funding acquisition, Supervision.

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Appendixes

Appendix 1. F values of the analysis of variance (two-way ANOVA) of growth and gas exchange parameters (A – photosynthetic rate, g_s – stomatal conductance, E – transpiration rate, WUE – water use efficiency) of rape and wild mustard exposed to interspecific competition (Comp), ozone (O_3) and future climate (Climate) treatments as well as their combinations.

	Plant	Shoot DW	Root DW	Leaf area	*A*	g_s	*E*	WUE
Comp	Rape	4.8*	26.8***	21.8***	8.4*	12.5**	8.9*	9.1*
Climate		28.1***	17.4***	16.1**	29.7***	42.2***	261.2***	83.3***
O_3		0.8^{ns}	1.2^{ns}	4.9*	47.8***	167.0***	142.0***	0.3^{ns}
Comp*Climate		4.2^{ns}	3.0^{ns}	20.9***	2.0^{ns}	0.2^{ns}	0.6^{ns}	10.3**
Comp*O_3		0.6^{ns}	2.9^{ns}	1.5^{ns}	3.0^{ns}	0.7^{ns}	1.5^{ns}	0.2^{ns}
Climate*O_3		0.3^{ns}	11.2**	4.8*	0.6^{ns}	1.2^{ns}	12.3**	7.4*
Comp*Climate*O_3		0.1^{ns}	6.2*	0.3^{ns}	0.3^{ns}	5.4*	3.8^{ns}	5.6*
Comp	Wild mustard	59.1***	16.3***	11.9***	28.0***	16.5***	21.4***	5.0**
Climate		81.3***	1.2^{ns}	12.5**	55.3***	116.1***	490.5***	114.8***
O_3		4.1^{ns}	5.1*	12.7**	28.5***	135.2***	110.5***	0.8^{ns}
Comp*Climate		20.4***	6.5**	3.8*	6.8**	4.7**	8.5***	2.5^{ns}
Comp*O_3		2.6^{ns}	4.6**	2.2^{ns}	1.5^{ns}	0.7^{ns}	0.7^{ns}	0.8^{ns}
Climate*O_3		22.2***	14.3***	16.6***	10.2**	2.3^{ns}	12.1**	21.9***
Comp*Climate*O_3		2.4^{ns}	8.2***	0.2^{ns}	0.5^{ns}	1.5^{ns}	1.5^{ns}	0.4^{ns}

ns Not significant.
*$p < 0.05$.
**$p < 0.001$.
***$p < 0.0001$.

Appendix 2. F values of the analysis of variance (two-way ANOVA) of biochemical parameters (Chl a + b – chlorphyl a and b, Carot – carotenoids, TSS – total soluble sugars, MDA – malondilhehyde, TAC – total antioxidative capacity) of rape and wild mustard exposed to interspecific competition (Comp), ozone (O_3) and future climate (Climate) treatments as well as their combinations.

	Plant	Chl a + b	Carot	TSS	MDA	TAC	Poly-phenols	Flavo-noids
Comp	Rape	13.3**	0.0^{ns}	1.7ns	8.5**	10.3**	8.0*	9.4**
Climate		38.6***	65.0***	38.0***	53.9***	776.8***	360.9***	101.2***
O_3		1.3^{ns}	0.5^{ns}	0.3ns	2.0^{ns}	5.5*	13.0**	22.8***
Comp*Climate		12.2**	15.3**	0.2ns	25.3***	20.9***	16.9***	76.4***
Comp*O_3		25.2***	11.4**	12.3**	56.1***	8.2*	39.5***	0.5^{ns}
Climate*O_3		3.1^{ns}	6.5*	18.7***	3.5^{ns}	0.7^{ns}	1.0^{ns}	24.6***
Comp*Climate*O_3		0.9^{ns}	10.7**	2.3ns	1.4^{ns}	4.2^{ns}	0.4^{ns}	63.6***
Comp	Wild mustard	10.9***	12.2***	0.4ns	0.4^{ns}	7.9***	2.9^{ns}	10.9***
Climate		18.2***	48.3***	72.1***	49.1***	769.2***	137.8***	154.9***
O_3		15.0***	10.8**	1.4ns	0.7^{ns}	0.2^{ns}	0.8^{ns}	1.7^{ns}
Comp*Climate		9.0***	7.3***	0.3ns	8.6***	3.4*	8.6***	17.5***
Comp*O_3		1.7^{ns}	1.5^{ns}	9.7***	3.4*	1.1^{ns}	6.8**	2.9^{ns}
Climate*O_3		23.1***	27.3***	6.2**	46.8***	1.4^{ns}	9.4**	76.8***
Comp*Climate*O_3		1.5^{ns}	0.9^{ns}	5.1**	3.8*	2.9*	2.3^{ns}	8.3***

ns Not significant.
*$p < 0.05$.
**$p < 0.001$.
***$p < 0.0001$.

Appendix 3. F values of the analysis of variance (two-way ANOVA) of antioxidative enzymes (CAT – catalase, POX – peroxidase, APX – ascorbate peroxidase, DHAR – dehydroascorbate reductase, MDAR – monodehydroascorbate reductase, GR – glutathione reductase, GPX – glutathione peroxidase) of rape and wild mustard exposed to interspecific competition (Comp), ozone (O_3) and future climate (Climate) treatments as well as their combinations.

	Plant	CAT	POX	APX	DHAR	MDAR	GR	GPX
Comp	Rape	0.0^{ns}	52.9***	66.9***	0.0^{ns}	0.0^{ns}	0.2^{ns}	29.1***
Climate		0.9^{ns}	5.8*	5.0*	5.9*	0.5^{ns}	4.7*	0.2^{ns}
O_3		4.4^{ns}	37.9***	57.0***	1.7^{ns}	2.4^{ns}	0.1^{ns}	3.7^{ns}
Comp*Climate		2.1^{ns}	0.4^{ns}	7.2*	4.5^{ns}	4.2^{ns}	13.8**	0.6^{ns}
Comp*O_3		4.0^{ns}	12.7**	26.2***	8.6**	14.1**	2.5^{ns}	7.0*
Climate*O_3		1.4^{ns}	8.0*	1.5^{ns}	1.2^{ns}	1.8^{ns}	4.2^{ns}	0.6^{ns}
Comp*Climate*O_3		4.7*	2.7^{ns}	0.7^{ns}	0.3^{ns}	1.7^{ns}	0.4^{ns}	23.3***
Comp	Wild mustard	0.1^{ns}	7.3*	10.7**	12.5**	3.4^{ns}	3.9^{ns}	21.7***
Climate		1.1^{ns}	4.1^{ns}	5.1*	3.0^{ns}	14.9**	4.5^{ns}	0.0^{ns}
O_3		3.2^{ns}	8.8**	12.2**	14.4**	0.0^{ns}	1.4^{ns}	13.7**
Comp*Climate		0.3^{ns}	9.4**	14.1**	0.0^{ns}	18.4**	2.4^{ns}	7.3*
Comp*O_3		2.2^{ns}	3.2^{ns}	6.2*	0.1^{ns}	36.0***	1.2^{ns}	8.8**
Climate*O_3		1.1^{ns}	3.9^{ns}	7.7*	6.6*	26.2***	0.0^{ns}	3.7^{ns}
Comp*Climate*O_3		8.2*	7.3*	10.4**	16.0**	0.9^{ns}	2.6^{ns}	33.9***

^{ns} Not significant.
*$p < 0.05$.
**$p < 0.001$.
***$p < 0.0001$.

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