Interspecific plant competition mediates impact of climate change on the ecophysiology of a plant-herbivore interaction.

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Abstract

Biotic interactions shape community evolution, but we lack mechanistic insights into how physiological and ecological processes under climate change are altered by biotic interactions.

We used a two-trophic model community consisting of the aphid *Dysaphis plantaginea* feeding on the forb *Plantago lanceolata*, and a grass competitor *Lolium perenne* that does not experience herbivory by the aphid. Monocultures and mixtures were exposed to three relevant simulated climate scenarios.

Elevated CO_2 reduced the nitrogen content quality of *P. lanceolata*, while simultaneous increases of CO_2 and temperature modified the plant metabolomic component in different directions. Elevated CO_2 enhanced defense systems in *P. lanceolata*, but these effects were not altered by warming. Interspecific plant competition did, however, neutralise these responses. There were no indications for indirect effects of climate on aphid performance as mediated by changes in plant defense, nutritional quality and biomass.

We thus demonstrate interactions between abiotic and biotic processes on plant metabolite profiles, but more importantly, that climate change effect on a selection of the metabolic pathways are altered by herbivory and intraspecific competition. Our experiment under semi-natural conditions thus demonstrates the non-additive and often neutralizing effects of biotic interactions on plant physiology and ecology under climate change.

Introduction

Interactions between insect herbivores and host plants are influenced by primary and secondary metabolites. The concentrations and types of these may, in turn, be affected by climate change (Bidart-Bouzat & Imeh-Nathaniel, 2008; Zvereva & Kozlov, 2006). Plants exposed to a CO_2-enriched environment show higher concentrations of carbohydrates (including starch and soluble sugars), lower concentrations of nitrogen (either from dilution by increased carbohydrates or reallocation) and thus a higher C:N ratio (Bezemer & Jones, 1998; Lincoln *et al.*, 1993; Robinson *et al.*, 2012; Stiling & Cornelissen, 2007). Lower nitrogen concentration implies lower levels of leaf protein and amino acids and, as a result, reduced nutritive value for herbivores (Lincoln *et al.*, 1986). Consequently, in general, the performance of insects reduces under high CO_2 (Bezemer & Jones, 1998; Hunter, 2001; Lincoln *et al.*, 1993). Contrary to elevated CO_2, warming has a direct positive effect on insect herbivore performance by reducing their development time (Bale *et al.*, 2002; Van De Velde *et al.*, 2016) and increasing fecundity (Meisner *et al.*, 2014) up to a certain threshold. Therefore, warming may indirectly compromise insect herbivores via their host plant quality (Bauerfeind & Fischer, 2013; Jamieson *et al.*, 2015). Nevertheless, such indirect effects are unlikely to fully counterbalance the direct positive effects, and the overall herbivore response to warming will be positive (Bauerfeind & Fischer, 2013; Zvereva & Kozlov, 2006).

Secondary metabolites (e.g. lignin, tannins, phenolics and terpenoids), affect tissue quality by altering the nutritive value, palatability, digestibility and/or toxicity of foliage. Compared to primary metabolites, the response of secondary metabolites to elevated CO_2 and warming is highly variable and less understood (Bidart-Bouzat & Imeh-Nathaniel, 2008; Robinson *et al.*, 2012). According to the carbon-nutrient balance hypothesis (Bryant *et al.*, 1983), elevated CO_2 is expected to increase carbon-based secondary compounds as a result of the ‘excess’ carbon (fertilizing effect) and to decrease nitrogen-based compounds a result of the nitrogen

scarcity (Robinson *et al.*, 2012). On the other hand, the growth-differentiation balance hypothesis (Herms & Mattson, 1992) proposes that warming-accelerated photosynthesis should contribute to growth rather than defense if resources (e.g. soil moisture and nutrients) are not limited. However, so far, studies suggest that the effects of warming and elevated CO_2 on the different groups of chemical defenses are idiosyncratic (Bidart-Bouzat & Imeh-Nathaniel, 2008; Zavala *et al.*, 2013; Zvereva & Kozlov, 2006).

Additional complexity arises from competition with neighboring plants. For example, competition for limited nutrients enhances the availability of carbon in a focal plant relative to its demand. This, in turn, can increase carbon-based defenses and thus reduce herbivory compared with control plants that are less constrained by competitors (Bryant *et al.*, 1983). Also the identity of a neighboring plant may affect the resistance of a focal plant by influencing the outcome of plant-plant competition (Barton & Bowers, 2006; Broz *et al.*, 2010). Conspecific competition, in particular, may result in stronger decline of plant growth and increased defense compared to heterospecific competition. Indeed, Broz *et al.* (2010) for instance showed that focal plants with conspecific neighbors allocated more resources towards production of carbon-based defense molecules, whereas those grown with heterospecific neighbors allocated more resources towards growth. Hence, elevated CO_2 as well as warming is anticipated to indirectly affect the defense of a focal plant, through species specific stimulation of plant growth.

Although atmospheric CO_2 and temperature increase concurrently, empirical studies investigating their combined effect on multi-trophic communities are surprisingly scarce and insufficient to effectively guide theory or synthesis (Cornelissen, 2011; Zvereva & Kozlov, 2006). The current study investigates individual effects of elevated CO_2 and combined effects of elevated CO_2 and warming on a simple model community consisting of three species: rosy apple aphid *Dysaphis plantaginea* Passerini (*Hemiptera: Aphididae*) feeding on plantain,

Plantago lanceolata L., and a heterospecific neighboring plant species, perennial ryegrass, *Lolium perenne* L. The aphid does not feed on *L. perenne.* The experimental design consisted of monocultures of *P. lanceolata* and mixtures of *L. perenne* and *P. lanceolata* exposed to three simulated climate scenarios. We hypothesized that (1) elevated CO_2 and warming alter foliar nutrients and defense molecules, (2) altered host quality and plant resistance affect insect herbivore performance, (3) elevated CO_2 and warming indirectly influence host quality and plant resistance via effects on neighboring plants.

Materials and methods

Experimental set-up

The study was performed at the Drie Eiken Campus, University of Antwerp, Wilrijk, Belgium (51° 09' N, 04° 24'E) in 12 sunlit, south-facing, climate-controlled chambers (details in Naudts *et al.* 2014). Three climate scenarios (four chambers per scenario) were simulated in an additive design: (1) current atmospheric CO_2 concentration and temperature (C); (2) future atmospheric CO_2 and current temperature (CO_2); and (3) future atmospheric CO_2 and temperature (TCO_2). Climate scenarios with elevated CO_2 had a target CO_2 concentration of 620 µmol mol^{-1} and future temperature chambers simulated a continuous 3 °C warming above fluctuating ambient temperatures. Climate manipulations were based on the IPCC-SRES B2-scenario prediction of moderate change for the year 2100 (IPCC, 2001).

The CO_2 concentration in each chamber was continuously measured and maintained at the target concentration with a CO_2 control group with an infrared analyzer (WMA-4, PPSystems, Hitchin, UK). Air temperature and relative humidity were monitored every 0.5 h with a combined humidity–temperature sensor (Siemens QFA66, Erlangen, Germany), by averaging instantaneous readings in half-hour mean values. During the experiment, the CO_2 concentration was 382 ± 55 µmol mol^{-1} (SD) in the current climate, while it was 615 ± 70 µmol mol^{-1} (SD) in the climate scenarios with future CO_2 concentration (CO_2 and TCO_2). The monthly average air temperature in the C and CO_2 chambers was 16.2, 17.2 and 18.7 °C in June, July and August, respectively. TCO_2 chambers were 2.9 ± 1.0 °C (SD) warmer than current temperature chambers. Average vapor pressure deficit was 0.60 ± 0.34 and 0.64 ± 0.52 kPa (SD) in the climate treatments with ambient and warmed air, respectively. Irrigation mimicked the rainfall outside (Naudts et al. 2014), with monthly totals equalling 64.4, 85.1 and 80.2 mm in June, July and August, respectively. Water freely drained while capillary rise

was prevented by a drainage system placed below the chambers. Future climate chambers (TCO_2) received the same amount of water as current climate chambers, so that any increase in water consumption would result in (aggravated) soil drought.

Plant and insect communities

We used two common co-occurring grassland species, *L. perenne* and *P. lanceolata*. *P. lanceolata* is characterized by the presence of the iridoid glycosides aucubin and catalpol. These compounds stimulate feeding and oviposition by specialist insects and can act as defense against generalist herbivores (Bowers & Puttick, 1988; Puttick & Bowers, 1988). Both plant species were sown at the end of March in a non-climate controlled greenhouse with a time lag of one week to prevent size differences at the start of the experiment (Cotrufo & Gorissen, 1997), and were watered twice a week. Four or five-week-old seedlings were transplanted into PVC containers (24 cm inner diameter and 40 cm height), filled with sandy soil (93.9% sand, 4.1% silt, 2.0% clay; pH 7.5; Kjeldahl-N 0.125 g kg^{-1}; 2.1% C in humus). Each of the 12 chambers received 20 containers with two different compositions: (1) 10 monocultures of *P. lanceolata*, and (2) 10 mixtures of both plant species in a 50:50 ratio. Each community contained 18 individuals planted in a hexagonal grid with 4.5 cm interspace. Interspecific interactions were maximized by avoiding clumping. All communities were fertilized with 10 g m^{-2} NH_4NO_3, 5 g m^{-2} P_2O_5, 10 g m^{-2} K_2O and micro-elements (Fe, Mn, Zn, Cu, B, Mo), given dissolved in water in two equal amounts.

When the plants were three months old, *P. lanceolata* was involuntarily infested with powdery mildew *Podosphaera plantaginis*. *P. plantaginis* is a biotrophic fungal pathogen which means that it feeds on living plant tissue, but does not kill the infected host. Biotrophic parasites extract nutrients from living cells and have extended periods of physiological

interaction with their hosts (Agrios, 2005). The powdery mildew was found in all climate scenarios and plant compositions but not all the containers were equally affected. Elevated CO_2 and warming have been shown to affect the severity of pathogen infections (Mikkelsen *et al.*, 2015, Thomas & Blanford, 2003). We took advantage of this unplanned infestation to study the impact of elevated CO_2 and warming on mildew infestation and the putative additive effects of mildew infestation on plant-insect herbivore interaction.

The rosy apple aphid *D. plantaginea* was used as an insect herbivore. It overwinters as eggs on apple trees, the primary host plant, and migrates in spring to the obligate alternate hosts, *P. major* L. and *P. lanceolata* (Alford, 2014). On *Plantago* spp., they give birth to apterous (wingless) morphs that reproduce by parthenogenesis (Blommers *et al.*, 2004). *L. perenne* is not a host plant for *D. plantaginea*. The aphids were reared in small cages on *P. lanceolata* under laboratory conditions of 22 ± 1 °C. They were introduced on *P. lanceolata* when the plants were 20 weeks old. At this time, two adult, apterous aphids were placed with a dry paintbrush on the apex of each *P. lanceolata* plant in monocultures and mixtures. Consequently, at the start of the infestation each container contained 36 (monocultures) or 18 (mixtures) aphids. In each chamber, four monocultures of *P. lanceolata* and four mixtures were randomly chosen for aphid infestation. Containers that did not receive aphids acted as controls. During infestation, all containers (both control and herbivory treatments) were individually enclosed with an 85-cm-tall cylinder of lightweight netting to ensure aphids did not migrate between pots. The infrastructure did not physically limit plant growth and did not cause photosynthetic stress effects (F_v/F_m, the intrinsic efficiency of PSII in controls = 0.84 which is an optimal value (Johnson *et al.*, 1993)).

Data collection

In the fourth week after the aphid introduction we determined the mildew infestation. The degree of powdery mildew on *P. lanceolata* was categorized by a rating system: 1) healthy (no visible lesions); 2) 1% - 25% of the leaves damaged; 3) 26% - 50% of the leaves damaged; 4) 51% - 75% of the leaves damaged; 5) more than 75% of the leaves damaged. At the same time, aphid populations of four replicate communities per plant composition in each chamber were collected (totaling 4 replicate communities × 2 plant compositions × 12 chambers). Aphids were brushed directly into 70% ethanol with a dry paintbrush. It was not possible to collect all the aphids because their number per container was either too high or too low (hard to find), therefore a subsample of the population per container was collected: we searched for and collected aphids for 30 minutes. The total number of aphids per community was divided by the number of *P. lanceolata* individuals in that community. At the same time, we also harvested the plants of two replicate communities per treatment in each chamber (totaling 2 replicate communities × 4 treatments × 12 chambers). Alive aboveground plant biomass in each community was separated from dead biomass by species, dried at 70 °C for 48 h and weighed. These biomass values per species were likewise divided by the number of the species' individuals in the community, providing primary data for the statistical analysis. The alive biomass samples of *P. lanceolata* plants of the two harvested communities per treatment in each chamber (2 replicate communities × 4 treatments × 12 chambers) were ground in a mill. Three subsamples per community were analyzed for nitrogen and carbon content using a NC element analyzer (NC-2100 element analyzer, Carlo Erba Instruments, Milano, Italy), which were averaged prior to data analysis.

A separate subsample of the milled live aboveground biomass of *P. lanceolata* of the two harvested communities per treatment in each chamber (2 replicate communities × 4 treatments × 12 chambers) was taken to quantify several biochemical parameters: fructose, sucrose, glucose, total soluble sugars, starch, total proteins, lipids, phosphorus, cellulose,

tannin, lignin, catalpol, aucubin, total phenols, tocopherols, carotenoids, total antioxidant capacity (TAC), jasmonic acid, salicylic acid, malondialdehyde (MDA) and proline (Appendix S1).

Data analysis

We focused on *P. lanceolata* because this is the host plant for the aphid and powdery mildew. In a first step we examined covariation in the metabolites responses to the three treatments (aphid infestation, climate scenario and plant composition) by using a hierarchical clustering analysis. Metabolites were normalized by Z-transformation and subjected to a hierarchical clustering analysis with an Euclidean distance metric, and visualized as a heat map using Multi Experiment Viewer (Mev) version 4.8 (Saeed *et al.*, 2003). A distance cut off of 0.3 was applied. The clusters were used in the structural equation models (see below).

In a second step, we fitted two piecewise Structural Equation Models (SEM), which combine information from multiple separate linear models into a single causal network (Shipley, 2009). The first SEM investigated the effect of aphid infestation, climate scenario, plant composition and mildew infestation on the metabolites and alive aboveground biomass of *P. lanceolata*. In a second SEM, we separated the metabolites of *P. lanceolata* in control pots from those with aphids. This allowed us to test the effects of the metabolites on the aphid population, as well as to quantify effects of the aphid population on these metabolites. The response of the aphid population was measured as their number at the end of the infestation. The four clusters obtained by the clustering analysis were used to divide the metabolites in separate groups. We standardized the metabolites, the alive aboveground biomass of *P. lanceolata* and the number of aphids by converting to Z-scores to equalize variances (see above). To reduce the number of mildew categories, we rearranged degree of mildew in two

categories: (1) no or mild mildew infection (category 1 - 2) (2) severe mildew infection (category 3 - 5).

Traditional SEM estimation methods assume that all variables follow a normal distribution and all observations are independent (Grace, 2006). In our analyses, we used piecewise SEM that allows fitting general linear mixed effect models that can incorporate random effects. Each mixed effects model was fitted using the “lme” function in the “nlme” package (version 3.1-128) in R. For each model, we fitted a random effect of chamber. The overall path model (the SEM) was fitted using the “piecewiseSEM” package (version 1.2.1) in R (Lefcheck, 2016). Goodness of fit was estimated using Shipley’s test of d-separation, which yields a Fisher’s C statistic that is Chi-squared distributed (Shipley, 2009). If the resulting P-value > 0.05, then the SEM can be said to adequately reproduce the hypothesized causal network.

In a final step, all data were analyzed with General Linear Mixed models (GLM) in SAS (version 9.2, SAS Institute Inc., Cary, NC) (Littell *et al.*, 1996) with chamber as a random factor nested within climate scenario. Climate scenario, plant composition, aphid infestation and two-way and three-way interactions between these predictors were included as fixed factors. Because mildew infestation did not have a significant effect on biochemical plant responses, this treatment was excluded from the GLM analysis. Non-significant factors were backwards-excluded from the model. In case of significant effects, *a posteriori* means comparisons using the Tukey test, corrected for multiple comparisons, were made. Effects were considered significant at $P \leq 0.05$.

Results

Hierarchical clustering analysis of metabolites

The variation in primary and secondary metabolites of *P. lanceolata* was subjected to a hierarchical clustering analysis in order to understand their grouping according to their covariation in response to the imposed herbivory, climate and competition treatments. We obtained four major clusters of metabolites (1 - 4 in Fig. 1).

The first cluster containing metabolites consistently upregulated by the presence of aphids contains the phenol antioxidants, the lipophilic antioxidant tocopherol, the C:N ratio, tannins, and the non-structural carbohydrates soluble sugars (including glucose, fructose) and starch (Fig. 1). The second cluster contains another group of antioxidant molecules that are downregulated by elevated CO_2 and more so by aphids in mono-cultures, the carotenoids, as well as the growth regulator, jasmonic acid, total proteins, phosphorous and lipids (Fig. 1).

The nutrient N shows a unique pattern, not shared by other metabolites. Carbon concentration, the C:P and N:P ratio, and lignin:N were classified in the fourth cluster that is specifically upregulated by aphids in monocultures under elevated CO_2 (Fig. 1). Cluster 4 also contains metabolites that play a role in plant defenses against herbivores, such as salicylic acid and the iridoid glycosides (catalpol and aucubin), and also contains TAC, lignin, cellulose, proline and MDA. Notably in this cluster, metabolites are strongly induced in CO2 and TCO2.

Causal changes as inferred by structural equation models

SEM quantify the strength of both the direct and indirect interactions between the climate change scenarios, herbivory and plant phenotype. The first SEM presents the effect of aphid

infestation (qualitative effect), climate scenario, plant composition and mildew infestation on the chemical composition and the live aboveground biomass of *P. lanceolata* (Fig. 2A). In this analysis, the metabolites of *P. lanceolata* were subdivided into the four groups obtained from the hierarchical clustering. The hypothesized structural relationship as outlined at the end of the introduction adequately fits the data (χ^2 = 42.53, df = 32, p = 0.101). Fig. 2A and Table S2.1 (appendix S2) provide us with three insights. Firstly, TCO_2 increased the mildew infestation compared to C, but the mildew infestation, in turn, did not affect the metabolites and the alive aboveground biomass of *P. lanceolata*. Secondly, neither climate scenario, nor aphid infestation or plant composition had a significant impact on the live aboveground biomass of *P. lanceolata.* Thirdly, climate scenario, aphid infestation and plant composition impacted the distinguished clusters in a different manner. (see previous section and Fig. 1). We noticed a significant interaction between climate scenario and aphid infestation for metabolites associated to this cluster. Also the metabolites in cluster 2 (see previous section and Fig. 1) differed according to an interaction between Climate scenario and aphid infestation interactions also affected the cluster 2 metabolite values . However, the three treatments did not affect the metabolites in cluster 3 (see previous section and Fig. 1). Aphid infestation, CO_2 and TCO_2 increased the metabolites in cluster 4 (see previous section and Fig. 1). Besides these single factor treatments effects, climate scenario, aphid infestation and plant composition also interacted with each other in affecting the metabolites in cluster 4.

The second SEM presents the effect of climate scenario, plant composition and chemical composition of *P. lanceolata* on the aphid population and whether the aphid population, in turn, altered the chemical composition of *P. lanceolata* (quantitative effect). The mildew infestation was left out of this analysis since it is a parasite of the plants, not of the aphids.

The results of this SEM model show the following goodness of fit statistics: $\chi^2 = 77.52$, df = 80 and p = 0.558 (Table S1.1, S1.2 and Fig 2) provide us with two insights. Firstly, climate scenario, plant composition and the chemical composition of *P. lanceolata* did not affect the number of aphids. Secondly, the aphid population size in turn, did not change the chemical composition of *P. lanceolata*. Bringing together the findings from both SEM models, we conclude that the treatment aphid infestation altered the chemical composition of *P. lanceolata* but the number of aphids had no effect on it.

Detailed analyses of the separate paths by General Linear Mixed models

Climate scenario, but not plant composition or aphid infestation altered the live aboveground biomass of *P. lanceolata* ($F_{2,9} = 6.34$, p = 0.019; $F_{1.82} = 0.84$, p = 0.362; $F_{1.82} = 0.98$, p= 0.325, respectively, Fig. 3A). The live aboveground biomass tended to be higher in CO_2 than C and was significantly lower in TCO_2 than CO_2, which combined lead to similar values in TCO_2 and C. Plant composition, but not climate scenario or aphid infestation altered the dead aboveground biomass of *P. lanceolata* ($F_{1,75} = 72.82$, p = <0.001; $F_{2.9} = 0.93$, p = 0.428; $F_{1,75} = 0.71$, p = 0.401, respectively, Fig. 3B). Competition from *L. perenne* reduced the dead aboveground biomass relative to growing with conspecific neighbors. Furthermore, climate scenario and plant composition did not alter relative herbivory effects ($F_{2.42} = 0.02$, p = 0.981; $F_{1.42} = 2.37$, p = 0.131, respectively). In addition, climate scenario and plant composition did not alter the number of aphids per plant ($F_{2,9} = 0.45$, p = 0.650; $F_{1,78} = 0.87$, p = 0.353, respectively).

An overview of all GLM summary statistics for the metabolomics endpoints and figures of the mean (+SE) values are provided in supplementary material 2 (Tables S2.1-4). We here

synthesize the most prominent results, and visualize a selection of relevant metabolites in Fig. 4 .

Cluster 1 metabolites (Fig S2.1)

As a baseline, the aphid infestation increased the concentrations of the metabolites in cluster 1 irrespective of climate scenario and plant composition (except for C:N ratio) (Fig. 4A). However, the effect of aphid infestation on the concentration of tannin depended on a significant interaction with plant composition. Aphid infestation increased the concentration of tannin in monocultures but not in mixtures. The concentrations of glucose and fructose increased with aphid infestation only in C, while the concentration of total phenol increased with aphid infestation in C and TCO_2 but not in CO_2 (Fig. 4B). Moreover, TCO_2 strengthened the effect of aphid infestation on the concentration of total phenol. In addition, CO_2, compared to C, increased the concentration of starch and the C:N ratio (Fig. A). TCO_2, in turn, reduced the concentration of starch (except for mixtures with aphids) and the C:N ratio compared to CO_2. The concentrations of glucose and soluble sugars increased at elevated CO_2 but only in monocultures, while TCO_2 did not alter the concentrations of glucose relative to the CO2 treatment but decreased the concentrations of soluble sugars in monocultures (Fig. 4C). In general CO_2, compared to C, increased the concentration of tannin while TCO_2 did not impose further differences. Pairwise comparison revealed that in C the concentration of tannin was higher in mixtures compared to monocultures while the opposite was true in CO_2.

Aphid infestation increased the concentration of tocopherol in monoculture and mixture in C, whereas in CO_2 and TCO_2 the increase under aphid infestation was more noticeable in mixture. Furthermore, the concentrations of tocopherol in monocultures with aphids declined at CO_2 compared to C. Despite this, the concentrations were higher in mixtures without

aphids at TCO_2 compared to CO_2 and C. Furthermore, interspecific interactions between *P. lanceolata* and *L. perenne* increased the concentrations of total phenols, starch and the C:N ratio, but did not modify the concentration of fructose (Fig S2.1).

Cluster 2 metabolites (Fig S2.2)

The concentration of jasmonic acid in C increased with aphid infestation in monocultures and mixtures but aphid infestation in CO_2 and TCO_2 decreased it in monocultures (Fig. 4D). Interspecific interactions between *P. lanceolata* and *L. perenne* mitigated the negative effect of aphid infestation in these climate scenarios. Furthermore, aphid infestation decreased the concentration of proteins in mixtures in C but did not alter the concentrations in monocultures and mixtures in CO_2 and TCO_2 (Fig. 4E). In general, CO_2 compared to C decreased the concentration of proteins irrespective of the plant composition while aphid infestation and TCO_2, compared to CO_2, did not alter it. The concentration of lipids decreased with aphid infestation in monocultures and mixtures in all climate scenarios (except for mixtures in C). Also, CO_2, compared to C, decreased the concentration of lipids in monocultures without aphids and mixtures with aphids, but in TCO_2, compared to CO_2, this concentration increased again to similar levels as C in monocultures without aphids. For carotenoids, there was a significant interaction between aphid infestation and climate scenario (Table 2). Aphid infestation in C did not alter the concentration of carotenoids while aphid infestation in TCO_2 reduced it. This was mainly due to an increase in the concentration of carotenoids in TCO_2 in controls, compared to C and CO_2. Aphid infestation reduced the concentration of P in monocultures, but did not alter it in mixtures (Fig. 4D). Furthermore, also CO_2, compared to C, reduced the concentration of P and the concentration was further reduced in TCO_2.

Cluster 3 metabolites

In cluster 3, aphid infestation and interspecific plant interactions slightly reduced the leaf nitrogen content (Fig. 4G).

Cluster 4 metabolites (Fig S2.3 and Fig S2.4)

Aphid infestation, irrespective of the climate scenario considerably increased the concentration of the metabolites of cluster 4 (except carbon) but more in monocultures than in mixtures Table S2.4). In other words, interspecific plant interactions between *P. lanceolata* and *L. perenne* mitigated the effect of aphid infestation. However, the concentration of aucubin increased with aphid infestation in both monocultures and mixtures. Furthermore, the metabolites of cluster 4 (except cellulose, catalpol, aucubin, carbon and MDA) varied also according to a significant interaction between plant composition and climate scenario. Elevated CO_2, compared to CO_2, increased the metabolites of cluster 4, but this positive effect of a future climate was mitigated by interspecific plant interaction (Table S2.4). TCO_2, compared to CO_2, increased further the N:P and the C:P ratio in monocultures but did not alter the concentration of lignin, sucrose, salicylic acid, proline, TAC, and the ratio lignin:N. Climate scenario did not alter the concentration of catalpol and carbon.

CO_2 and TCO_2 strengthened (salicylic acid and lignin) or induced (proline) an effect of aphid infestation but only in monocultures (Fig. 4H). The concentrations of lignin and proline did not differ between CO_2 and TCO_2 while the concentration of salicylic acid increased in monocultures with aphids in TCO_2 compared to CO_2. For cellulose, the three-way interaction between climate scenario, plant composition and aphid infestation was mainly due to a significant increase in the concentration of cellulose in monocultures with aphids in C compared to other treatments. In addition, CO_2 and TCO_2 also strengthened the effect of

aphid infestation on the concentration of aucubin but irrespective of the plant composition. Furthermore, TCO_2 increased the concentration of MDA compared to the other climate scenarios and this was most clear in monocultures with aphids.

Discussion

Our semi-natural experimental approach demonstrated that aphid herbivory induced resistance against oxidative stress and herbivory in *P. lanceolata*, thereby reducing biomass loss. Elevated CO_2 increased specific defense molecules and enhanced the effect of aphid herbivory on these molecules. However, warming did not alter these effects of CO_2. Furthermore, elevated CO_2 reduced the leaf quality for insect herbivores irrespective of the plant composition while aphid infestation and TCO_2, compared to CO_2, did not alter it.

Notwithstanding the effects of a future climate on leaf quality and defense molecules, we found no indications for indirect effects on aphid performance and herbivory. More importantly, we shod that interspecific plant competition neutralises the positive effects of elevated CO_2 on the production of defense molecules in *P. lanceolata* were neutralized. Induced effects of climate change can thus be altered and even equalised by biotic interactions. We first discuss the general herbivore-induced responses followed by an in-depth discussion on their dependency on the imposed climate and competition treatments.

Herbivore-induced responses

Our experiment, as conducted under unique semi-natural conditions confirmed in first instance findings that aphid infestation is reducing N, P, total proteins and lipids in *P. lanceolata* leaves thereby impairing nutritional requirements of insect herbivores(Schoonhoven *et al*., 2005) .In contrast to previous research (Walling, 2000), aphid infestation led to an increase in salicylic acid and jasmonic acid pathway (Kuśnierczyk *et al.*, 2011; Morkunas & Gabryś, 2011) as parallelized by the increased production of catalpol, aucubin, tannin, lignin and cellulose.

Aphid herbivory increased levels of ROS in plants as well (Wu & Baldwin, 2010). We assessed the induction of oxidative stress by measuring MDA, which aphid herbivory increased considerably. A common defense response of plants against oxidative stress entails changes in ROS scavenging antioxidant metabolites. Indeed, TAC and the well-known antioxidant molecules phenols and tocopherols increased due to aphid herbivory. Moreover, *P. lanceolata* responded to herbivory by increasing non-structural carbohydrates including starch and soluble sugars. At higher concentrations, also sugars can act as ROS scavengers or they can have a signaling function in regulating stress and defense responses (Gómez-Ariza *et al.*, 2007, Peshev *et al.*, 2013). Herbivory and the induced resistance against oxidative stress appeared sufficiently effective to constrain biomass loss.

Climate dependency of the plant

Elevated CO_2 and temperature did not change the impact of herbivory effects on *P. lanceolate* biomass. This implies that, warming and CO_2 did not affect the net interaction strength between plants and herbivores under semi-natural conditions. In contrast to other studies (Van De Velde *et al.*, 2016), our experiment allows a profound analysis on the underlying physiological processes.

First, we found elevated CO_2 increased to increase starch, soluble sugars (in monocultures) and lowered leaf proteins, hereby increasing the C:N ratio. In addition, elevated CO_2 reduced P and consequently increased the C:P ratio (in monocultures), thereby reducing the plant's nutritive value to herbivores (Huberty & Denno, 2006; Lincoln *et al.*, 1986; Mattson, 1980; Schoonhoven *et al.*, 2005).We demonstrate here that these effects van be countered when elevated CO_2 is accompanied with a temperature rise. This contradicts findings based on a

limited set of primary metabolites (Murray *et al.* 2013), but urges to apply broader phenomic approaches to fully understand plant responses to climate change.

Second, elevated CO_2 increased the defense molecules lignin and tannin, but the level of induction after aphid infestation. The induction of plant chemical defenses (Bidart-Bouzat & Imeh-Nathaniel, 2008; Bidart-Bouzat *et al.*, 2005), is thus not consistent for all defense molecules, and are thus anticipated to render prediction on future plant-insect herbivore interaction highly complex (Agrawal, 1999). In our study, the higher C:N ratio reflected higher lignin and tannin levels. While there is a consensus that the carbon-nutrient-balance hypothesis fails as predictive hypothesis (Hamilton *et al.*, 2001; Lindroth, 2012), alternative hypotheses have been proposed that resource utilization for chemical defense is linked with photosynthesis, hormone regulation and the control of gene expression (Zavala *et al.*, 2017). We here provide evidence for this alternative mechanism as we found CO_2 to alter phytohormones (i.e., salicylic acid and jasmonic acid) that play an important role in promoting compounds responsible for herbivore defense (Wu & Baldwin, 2010). Since no further quantitative changes were observed under higher temperature, we can confidently conclude that climate-change induced changes in metabolic profiles are more due to enhanced CO_2 rather than the in parallel acting temperature increases.

Competition: a biotic interaction mediating climate-induced effects

The presence and identity of neighboring plants can influence the quality of the host plant by altering primary and secondary chemistry (Barton & Bowers, 2006; Broz *et al.*, 2010, Lankau, 2012; Thorpe *et al.*, 2011). We provide evidence that these biotic interactions are additionally able to alter climate-induced phenomic changes, and by that further impacting

ecological interactions under climate change. Energetic growth-defense-trade-offs have usually been attributed to these competition effects (Herms & Mattson, 1992), but recent studies demonstrated the role of light limitation for plant defense downregulation (Ballaré, 2014; Campos *et al.*, 2016). More specifically, a reduced red to far-red ratio leads to a negative regulation of defenses by a simultaneous inhibition of the jasmonic acid and the salicylic acid pathway (Wit *et al.*, 2013). The lower levels of defense molecules and salicylic acid in *P. lanceolata* support this hypothesis.. Competition did, however, also countered the CO_2 –induced changes in primary and secondary metabolites, as well as those induced by herbivores, rendering an understanding of plant defenses far from trivial (de Vries *et al.*, 2017). It is well-understood that bottom-up effects from plant quality to herbivore abundance cannot be generalized across the feeding guilds (Bezemer & Jones, 1998; Robinson *et al.*, 2012; Stiling & Cornelissen, 2007), but phloem feeders as aphids were at least expected to be facilitated. Despite the complex, high-dimensional changes in plant metabolomes under competition and climate change, the overall effects on aphid population sizes were small to non-existent.

We here thus demonstrate that the joint action of climate change and biotic interactions is able to induce pronounced changes in plant metabolomes and biomass, but that translation of these physiological changes towards ecological responses appears non-trivial. Climate-change imposed changes are shown to be completely neutralized when common biotic interactions are taken into consideration. Plant-plant interactions thus add a layer of complexity in mechanistic studies of climate change effects on plant-enemy ecological interaction.

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Supplementary data:

Supplementary material 1: Metabolite quantification methods and SEM statistics

Supplementary material 2: results from the univariate GLM analyses (Statistics and figures)

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Figure legends

Fig. 1 Heat map showing the metabolite levels in the leaves of *P. lanceolata*, normalized to Z-score for each metabolite (blue-white-red heat map). Red and blue colours indicate a low and high metabolite level, respectively. Clustering was based on the Euclidean distance for metabolites. Labels 1-4 and colors indicate the four prominent clusters. Labels C, CO_2 and TCO_2 indicate current climate, elevated CO_2 and combined warming and elevated CO_2, respectively. Plant communities consist of monocultures of *P. lanceolata* (mono) and mixtures of *Lolium perenne* and *P. lanceolata* (mix) with (A) and without aphids (C).

Fig. 2 (A) Structural equation model showing how climate scenario (CO_2 and TCO_2), mildew infestation, plant composition and aphid infestation affect the chemical composition and the live aboveground biomass of *P. lanceolata*. (B) Structural equation model showing how climate scenario, plant composition and chemical composition of *P. lanceolata* affect aphid population and how aphid population, in turn, affects the chemical composition of *P. lanceolata*. The four clusters refer to those obtained by the hierarchical clustering analysis (see Fig. 1). Solid black, green and orange arrows represent significant relationships ($P \leq 0.05$) and dashed grey lines significant interactions. Blue lines stand for significant effects of both CO_2 and TCO_2, green lines for significant effects of CO_2 and orange lines for significant effects of TCO_2. Light grey arrows represent non-significant relationships. Standardized path coefficients are shown next to pathways. For the effect of CO_2 and TCO_2, the average path coefficients are shown. The individual path coefficients of CO_2 and TCO_2 can be seen in Table S1 and Table S2 (see supplementary material section 2). Metabolites levels, live aboveground biomass of *P. lanceolata* and number of aphids were scaled before analysis.

Fig. 3 Effect of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition on the live (A) and dead (B) aboveground biomass of *Plantago lanceolata*. Bars represent means ± SE. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*.

Fig. 4 Effect of climate scenario (C, CO_2 and TCO_2), aphid infestation and plant composition on the on a selection of relevant metaboloites from the four distinguished clusters. Bars represent means ± SE. Plant communities consist of monocultures of *P. lanceolata* and mixtures of *Lolium perenne* and *P. lanceolata*.

Cluster 1: A: C:N ratio; B: total phenols; C: soluble sugars

Cluster 2: D: Jasmonic acid; E: phosphorous, F: total proteins.

Cluster 3: G leaf nitrogen fractions

Cluster 4: H: salicylic acide; I: TAC; J: N:P ratio; K: C:P ratio;

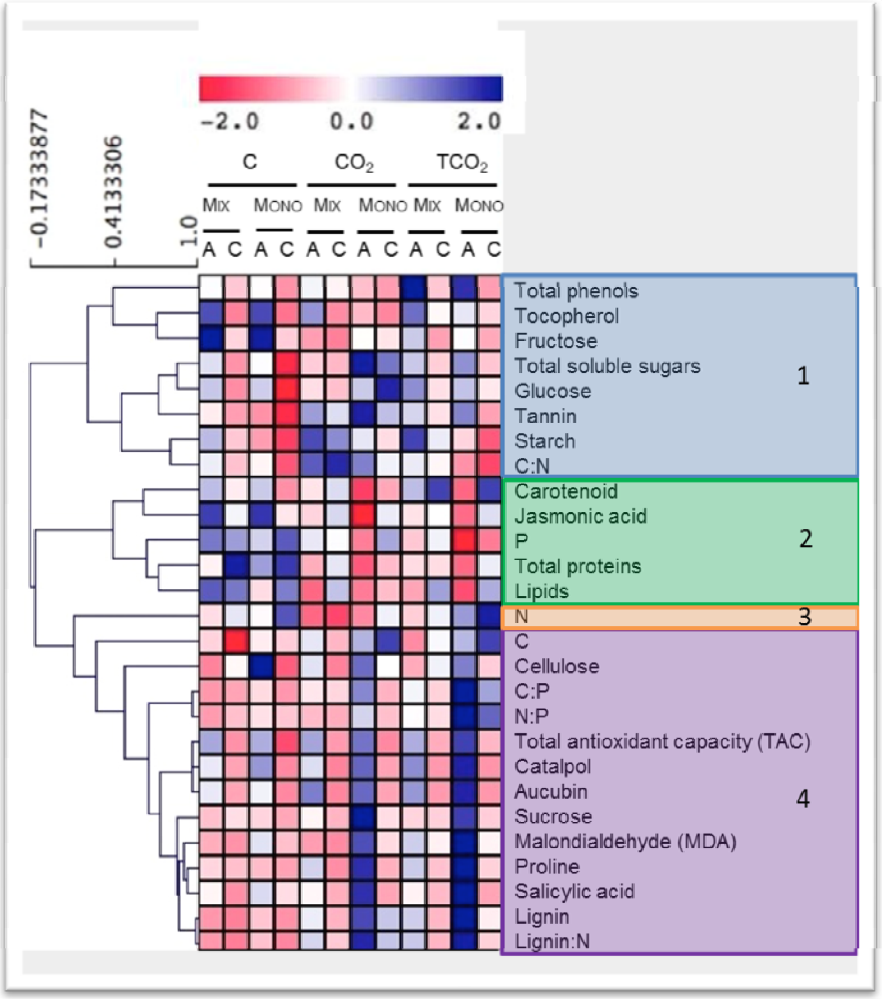

-2.0
0.0
2.0
C
CO2
TCO2
Mix
Mono
Mix
Mono
Mix
Mono
A C A C A C A C A C A C
-0.17333877
0.4133306
1.0
Total phenols
Tocopherol
Fructose
Total soluble sugars
Glucose
Tannin
Starch
C:N
1
Carotenoid
Jasmonic acid
P
Total proteins
Lipids
2
N
3
C
Cellulose
C:P
N:P
Total antioxidant capacity (TAC)
Catalpol
Aucubin
Sucrose
Malondialdehyde (MDA)
Proline
Salicylic acid
Lignin
Lignin:N
4

Fig. 1

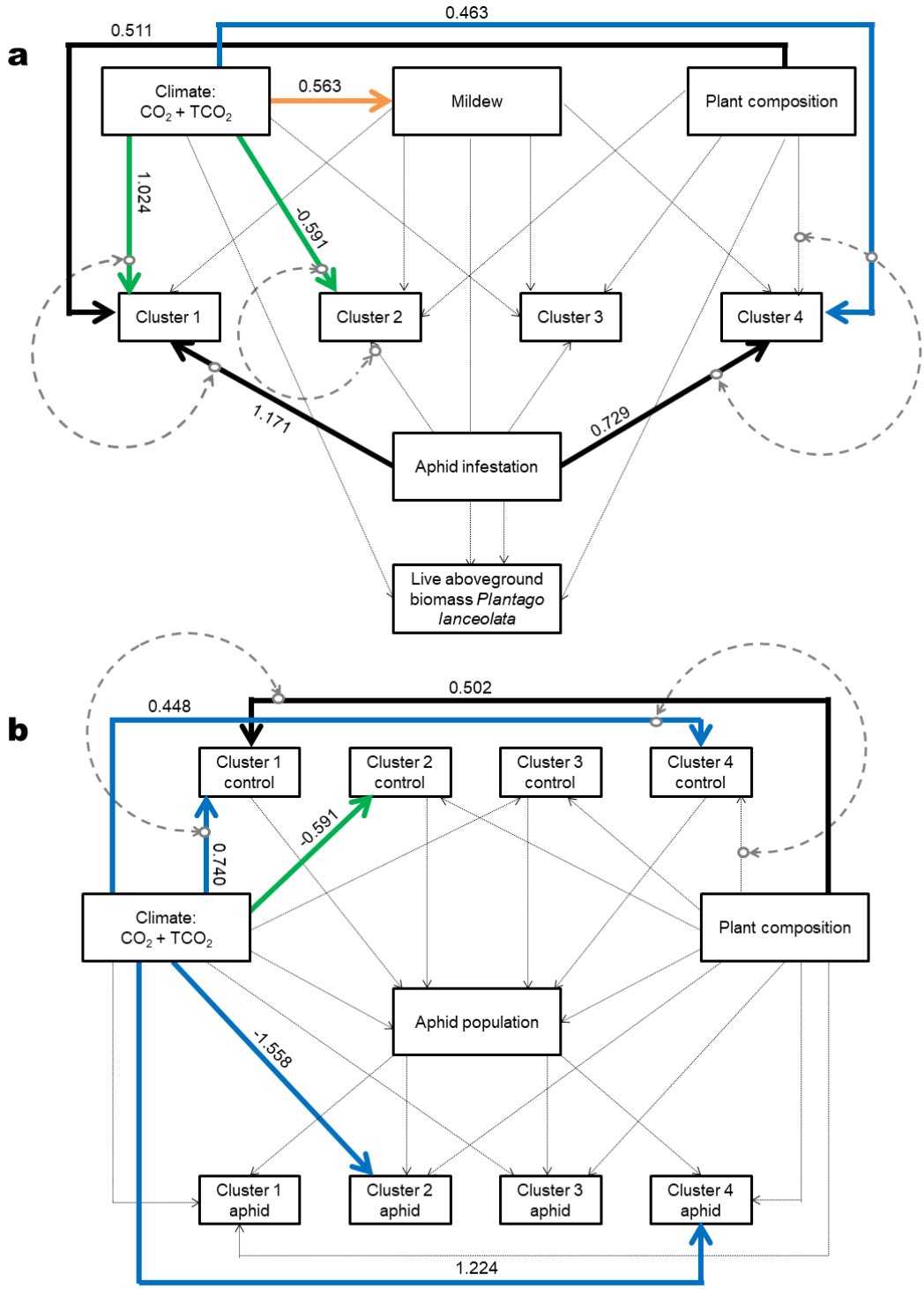

a
0.511
0.463
Climate:
CO_2 + TCO_2
0.563
Mildew
Plant composition
1.024
-0.591
Cluster 1
Cluster 2
Cluster 3
Cluster 4
1.171
0.729
Aphid infestation
Live aboveground biomass *Plantago lanceolata*
b
0.448
0.502
Cluster 1 control
Cluster 2 control
Cluster 3 control
Cluster 4 control
-0.591
0.740
Climate:
CO_2 + TCO_2
Plant composition
Aphid population
-1.558
Cluster 1 aphid
Cluster 2 aphid
Cluster 3 aphid
Cluster 4 aphid
1.224

Fig. 2

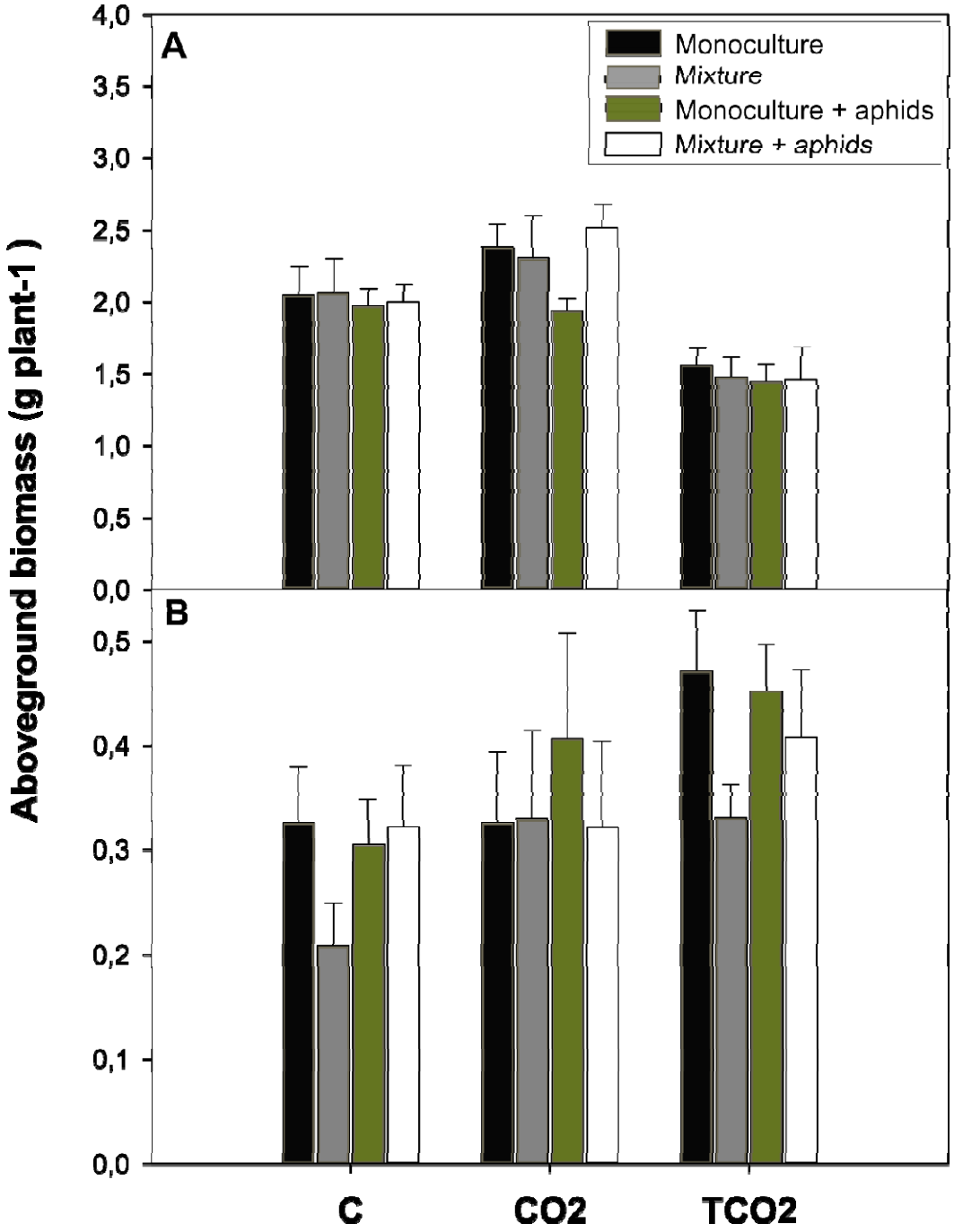
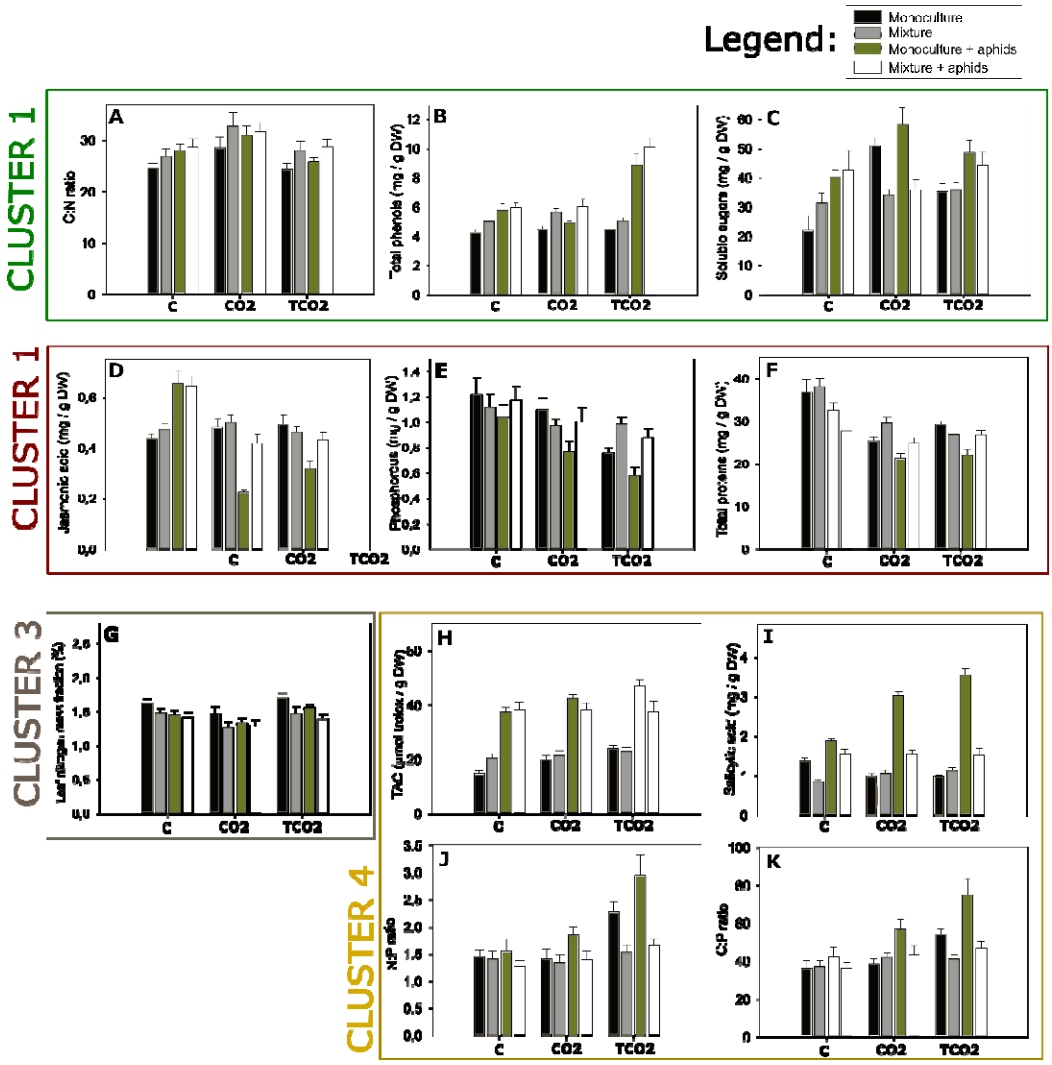

A
B
Monoculture
Mixture
Monoculture + aphids
Mixture + aphids
Aboveground biomass (g plant-1)
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0,5
0,4
0,3
0,2
0,1
0,0
C
CO2
TCO2

Fig. 3

Fig. 4


Legend:
Monoculture
Mixture
Monoculture + aphids
Mixture + aphids
CLUSTER 1
CLUSTER 1
CLUSTER 3
CLUSTER 4
A
B
C
D
E
F
G
H
I
J
K
C:N ratio
Total phenols (mg / g DW)
Soluble sugars (mg / g DW)
Jasmonic acid (mg / g DW)
Phosphorous (mg / g DW)
Total proteins (mg / g DW)
Leaf nitrogen (mass fraction (%))
TnC (µmol trolox / g DW)
Salicylic acid (mg / g DW)
N:P ratio
C:P ratio
C
CO2
TCO2