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Research Article

Elevated CO_2 alters photosynthesis, growth and susceptibility to powdery mildew of oak seedlings

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Elevated CO_2 (eCO_2) is a determinant factor of climate change and is known to alter plant processes such as physiology, growth and resistance to pathogens. *Quercus robur*, a tree species integrated in most forest regeneration strategies, shows high vulnerability to powdery mildew (PM) disease at the seedling stage. PM is present in most oak forests and it is considered a bottleneck for oak woodland regeneration. Our study aims to decipher the effect of eCO_2 on plant responses to PM. Oak seedlings were grown in controlled environment at ambient (aCO_2, ~400 ppm) and eCO_2 (~1000 ppm), and infected with *Erysiphe alphitoides,* the causal agent of oak PM. Plant growth, physiological parameters and disease progression were monitored. In addition, to evaluate the effect of eCO_2 on induced resistance (IR), these parameters were assessed after treatments with IR elicitor β-aminobutyric acid (BABA). Our results show that eCO_2 increases photosynthetic rates and aerial growth but in contrast, reduces root length. Importantly, under eCO_2 seedlings were more susceptible to PM. Treatments with BABA protected seedlings against PM and this protection was maintained under eCO_2. Moreover, irrespectively of the concentration of CO_2, BABA did not significantly change aerial growth but resulted in longer radicular systems, thus mitigating the effect of eCO_2 in root shortening. Our results demonstrate the impact of eCO_2 in plant physiology, growth and defence, and warrant further biomolecular studies to unravel the mechanisms by which eCO_2 increases oak seedling susceptibility to PM.

Introduction

Plants are continually exposed to multiple biotic and/or abiotic stresses that can negatively affect their growth, productivity and survival [1, 2]. Climate change, as a conjunction of carbon dioxide (CO_2) rise, temperature increase and frequent drought periods, alters plant physiology, adaptation and resistance to pathogens [3–5]. Due to the significance of woodland habitats for biodiversity there has been a robust effort to restore, manage and defragment existing woodlands through the use of landscape level management systems [6]. Understanding how the interaction of these factors affect plant resistance responses is crucial to determine management strategies and policies to protect natural forest environments.

Pedunculate oaks (*Quercus robur*) are one of the most economically and ecologically relevant forest tree species in Europe [7]. Oaks can have life spans exceeding 900 years and this longevity allows them to reach great sizes and to sequester large amounts of CO_2 [8]. Due to the economic and ecologic value of oaks, efforts are focusing on the regeneration of woodlands with this tree species. However, oak populations are facing several challenges that hinder their success, such as the combination of unfavourable climatic conditions and infection by pathogens [9]. One pathogen of particular relevance is the fungus *Erysiphe alphitoides,* causal agent of oak powdery mildew (PM) [10], which mainly affects young oak seedlings. *E. alphitoides* is a biotrophic pathogen present in Europe since 1907 [11] whose infection is characterised by white and cottony hyphae covering leaf surfaces, with

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necrosis and leaf deformation and loss at advance stage, resulting in a reduced functional leaf area and photosynthetic efficiency [12, 13]. Specifically, *E. alphitoides* causes increased transpiration rates while decreases CO_2 assimilation and carbohydrates translocation from infected leaves to the rest of the plant [13–16]. Whereas mature oak trees are also susceptible to PM, it has been demonstrated that seedlings show higher susceptibility, which has been suggested to be related to the differences in leaf traits between oak seedlings and mature trees: oak seedlings have a lower mass, a lower nitrogen content, a higher stomatal conductance and a higher rate of CO_2 assimilation than mature trees [17]. Usually, infections result in seedling death [18] and this is why this pathogen is considered to be a bottleneck to natural woodland regeneration [19]. There are reports as to the effect of climate on the severity of infections of PM in oak. For instance, it has been shown that higher humidity and temperatures of ∼22–25°C are beneficial to pathogen growth and wet weather in late summer are beneficial for the spreading of sexual spores [20]. There is also evidence that warmer winter temperatures increase rates of infection [7]. Therefore, weather conditions associated with climate change can have a deep impact on the severity of PM disease in oak, which warrants further studies on the effect of climate change in this pathosystem.

Current methods to control PM by tree nurseries and woodland managers depend on extensive application of chemical fungicides, which use is extremely limited due to their toxicity to human health and the environment [21]. Therefore, alternative methods to control this pathogen are needed. As described in many plant species, a plausible solution could rely on the exploitation of the plant's immune system through the activation of Induced Resistance (IR) and priming, the latter described as a sensitisation of defence mechanisms for a faster and/or stronger response upon subsequent stresses [22]. Priming can be achieved by different stimuli, including plant chemicals such as β-aminobutyric acid (BABA) [23, 24]. BABA has been shown to prime defence responses against a wide spectrum of biotic stresses [24–26] including powdery mildews in *Cucurbita* [27]. The broad-spectrum nature of BABA has been linked to the chemical's capacity to prime multiple defence mechanisms. For instance, BABA can prime SA-dependent defences such as gene expression and SA accumulation [28, 29] which result in resistance against biotrophic pathogens. In addition, BABA-IR has been shown to prime cell-wall defence through an effective deposition of callose at the infection sites [30, 31]. BABA priming of callose has been reported to be effective against both biotrophic and necrotrophic pathogens [25]. It is currently unknown whether BABA could offer a solution to protect oaks against PM. Nevertheless, it is important to consider that in agronomic settings, the use of BABA is not established as it has been shown to trigger a phytotoxic response that manifests mainly as a plant growth reduction [32, 33]. This phytotoxic response is due to the potential binding of BABA to its plant receptor [32] and to the transient relocation of energy from growth to defence [34]. It remains unknown whether BABA leads to a growth reduction in oak seedlings. Moreover, it is not understood whether the potential benefits from BABA treatment would outweigh the costs in growth in forest settings.

Associated with climate change is also the concentration of atmospheric CO_2, which has been on the rise since the 1960's [35]. Elevated levels of CO_2 (eCO_2) can drive deep changes in plant physiology and development [36] with different described responses among C3 and C4 plant species [37, 38]. For instance, it has been documented that eCO_2 results in major changes in growth at early stages of plant development, primarily driven by an increased efficiency of leaves to produce biomass [39] and increased N-use efficiency [40]. In addition, under eCO_2, starch concentrations more than double [41]. Therefore, eCO_2 might result in enhanced growth and productivity. Whilst this could be seen as a positive 'carbon fertiliser' effect, eCO_2 has also been associated to changes in the defensive capacity of plants that have proven contrasting. For instance, in soybean, it has been described that eCO_2 results in the production of phytoalexins and SA, resulting in enhanced resistance to pathogens [42, 43]. However, other studies testing resistance against fungal pathogens have described that eCO_2 does not impact resistance in ragweed [44] or even increases susceptibility in wheat [45] and maize [38]. Specific studies have been done to test the effect of eCO_2 in PM diseases. eCO_2 results in increased disease severity in Arabidopsis [46], soybean [47] or grapevine [48]. However, no effects were found in barley [49], or zucchini [50]. Interestingly, a study in Arabidopsis has reported that changes in the CO_2 concentration alter the capacity of plants to express IR [5]. Regarding the higher percentage of C3 plants (close to the 90%) versus the 1% of C4 [51, 52], it is not surprising that most of the literature is focused on C3 species, and scarce references about the effect of eCO_2 on resistances in C4 are available [38]. Furthermore, the effect that eCO_2 has in the severity of PM and expression of IR in oak remains to be studied.

Despite the extensive knowledge on the energy relocation trade-off between plant growth into defence [53, 54], little is known whether an increase in growth can also divert energy away from the activation of

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resistance responses. Nevertheless, it could be hypothesised that eCO_2 would increase growth through the production of young tender leaves, which could be more susceptible to the fungus [12], thus resulting in a detrimental effect. In this study, a range of physiological parameters and disease phenotypes have been linked to changes in growth triggered by eCO_2 and BABA, thus assessing the impact of eCO_2 on oak PM infections and IR.

Results

Effect of elevated CO_2 on growth and disease resistance

Seedlings grown under eCO_2 were taller than the ones growing at aCO_2 until 2 weeks of growth when heights stabilised (Figure 1A; Supplementary Figure S1A). Experiments revealed that plants growing under eCO_2 presented a higher number of PM colonies (Figure 1B; Supplementary Figure S1B) and a higher number of affected leaves (i.e. presenting colonies) (Figure 1C). Therefore, eCO_2 results in enhanced initial plant growth and susceptibility to PM.

Effect of β-aminobutyric acid (BABA) on growth and induced disease resistance

Seedlings treated with BABA presented similar height values than plants treated with water (Figure 2A). Treatments with BABA resulted in fewer colonies (Figure 2B) and a reduced number of affected leaves (Figure 2C) than water-treated plants. Therefore, BABA enhances resistance against PM while not reducing plant growth.

Effect of eCO_2 on BABA-induced resistance against powdery mildew

To assess the impact of eCO_2 on BABA-induced resistance (IR) against PM infection, plants growing at aCO_2 and eCO_2 were treated with water or BABA one week before infection with PM. To have plants of similar developmental stages upon treatment and infection, eCO_2 plants were germinated a week later [5]. As seen before, under aCO_2, BABA induces resistance against PM as BABA-treated plants have a lower number of colonies and leaves affected (Figure 3A,B). eCO_2 treatment in this particular experiment resulted in a dramatic enhanced susceptibility to PM that manifested as a high number of leaves being discarded by the plant (Figure 3C — 'lost' leaves). As a consequence, the number of colonies and affected leaves in infected plants under eCO_2 was lower than the aCO_2 plants (Figure 3A,B). Water-treated seedlings discarded leaves under both CO_2 conditions and BABA treatments fully protected from severe disease with no discarded leaves observed (Figure 3C and Supplementary Figure S3). Interestingly, eCO_2-grown plants treated with BABA had a higher number of colonies and affected leaves than aCO_2-grown BABA treated plants, however these observations were not statistically significant (Supplementary Figure S3). Therefore, BABA-induced resistance is maintained under eCO_2 but our results suggest that the treatment may not be as effective under eCO_2 as it is under aCO_2.

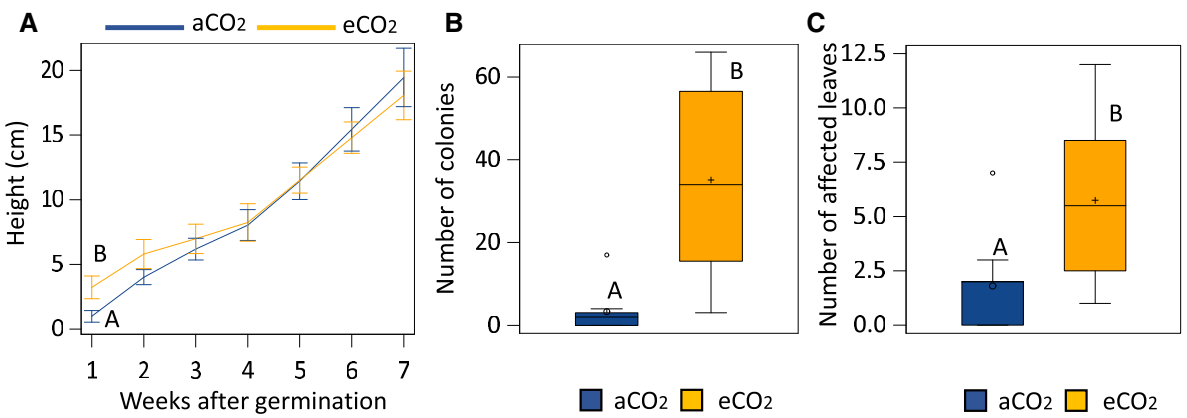

Figure 1. Effects of eCO_2 on growth (**A**) and PM resistance represented by the number of colonies per plant (**B**) and the number of leaves affected (i.e. presenting colonies) (**C**) at 14 days post infection (dpi). Capital letters represent statistically significant differences between CO_2 concentrations (Tukey post-hoc test; $P < 0.05$; $n = 8$).

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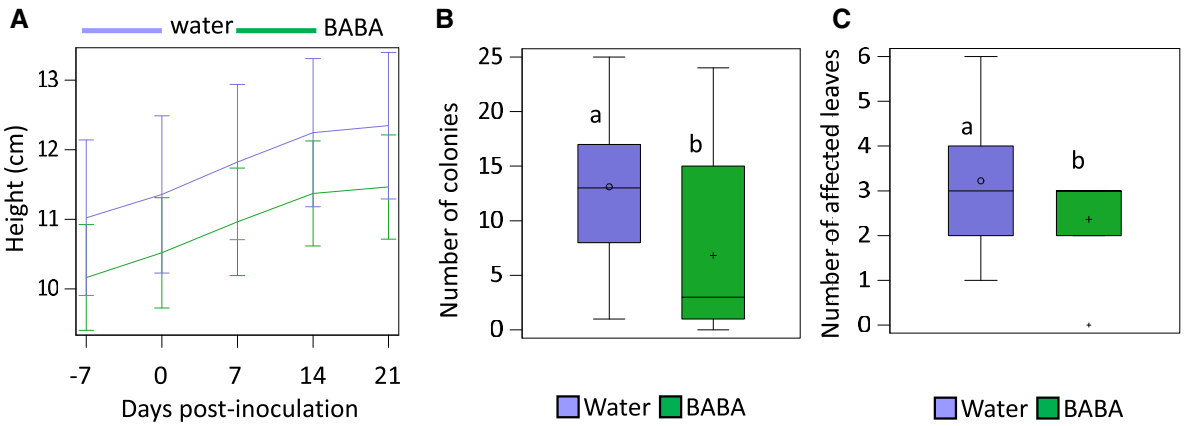

Figure 2. Effects of BABA treatment on growth (**A**) and PM resistance represented by the number of colonies per plant (**B**) and the number of affected leaves (i.e. presenting colonies) (**C**) at 14 days post infection (dpi). Lowercase letters represent statistically significant differences between BABA and water treatments (Tukey post-hoc test; $P < 0.05$; $n = 8$).

Effects of eCO_2 and BABA treatment on physiological parameters

Photosynthesis rate was measured in terms of net carbon assimilation ratio (An). We found that eCO_2 overall significantly increased An of infected seedlings throughout the experimental period (Figure 4A) compared to aCO_2. However, non-inoculated seedlings did not show differences in An between CO_2 levels in T2 and T3. Under eCO_2 levels, differences between BABA/water treatment and inoculated/non-inoculated plants were not detected (Figure 4A), indicating a saturation of the photosystems. However, under aCO_2, significant differences were found between BABA and water-treated seedlings as well as with the non-inoculated seedlings: BABA-treated seedlings maintained higher An than water-treated seedlings and in T3, non-inoculated plants had the highest An (Figure 4A).

Stomatal conductance was measured in all treatments. In contrast with the results observed for the An, seedlings growing under eCO_2 had a trend of lower values of stomatal conductance (Figure 4B). This observation was more pronounced in non-inoculated seedlings in T2 and T3. Under eCO_2, no statistically significant differences were found between treatments. Under aCO_2 conditions, similar profiles to those for An were observed, with non-inoculated plants showing the higher stomatal conductance, followed by inoculated plants treated with BABA and then inoculated plants treated with water (Figure 4B).

Minor differences were found for maximum photochemical efficiency of photosystem II (Fv/Fm) with the different concentrations of CO_2: plants growing under eCO_2 had slightly lower levels of Fv/Fm (Figure 4C). However, no significant differences were found at either treatment or infection profile (Figure 4C).

Effects of eCO_2 and BABA treatment on growth

Height, main stem diameter, root length and root and shoot biomass were measured to test the effect of eCO_2 and BABA on growth. eCO_2-grown plants were germinated one week after aCO_2-grown plants to work with plants at similar developmental stages guided by results presented in Figure 1A. Accordingly, no differences in height were observed between aCO_2 and eCO_2 plants (Figure 5A). However, height RGR (HRGR) revealed differences throughout the experiment. Firstly, between T1 and T2, both water and BABA-inoculated plants showed higher HRGRs under eCO_2 in comparison with their respective controls growing under aCO_2. Second, this effect was more pronounced in water treated plants under eCO_2 as they had a higher HRGR which was statistically significant compared with the HRGRs of BABA and non-inoculated plants. Third, as the experiment progressed, HRGRs decreased in all treatments apart from in plants treated with BABA and grown under aCO_2 (Figure 5B). Similarly to the height measurements, there were not statistically significant differences between aCO_2 and eCO_2 diameters (Figure 5C). Within CO_2 concentrations, BABA-treated plants inoculated with PM had bigger diameters than the water-treated plants and non-inoculated plants at T1 and T2. At T3 no differences in diameters were found between treatments. RGR values of the diameter (DRGR) showed that those observed under eCO_2 were higher between T2 and T3 in non-inoculated plants and in water-treated

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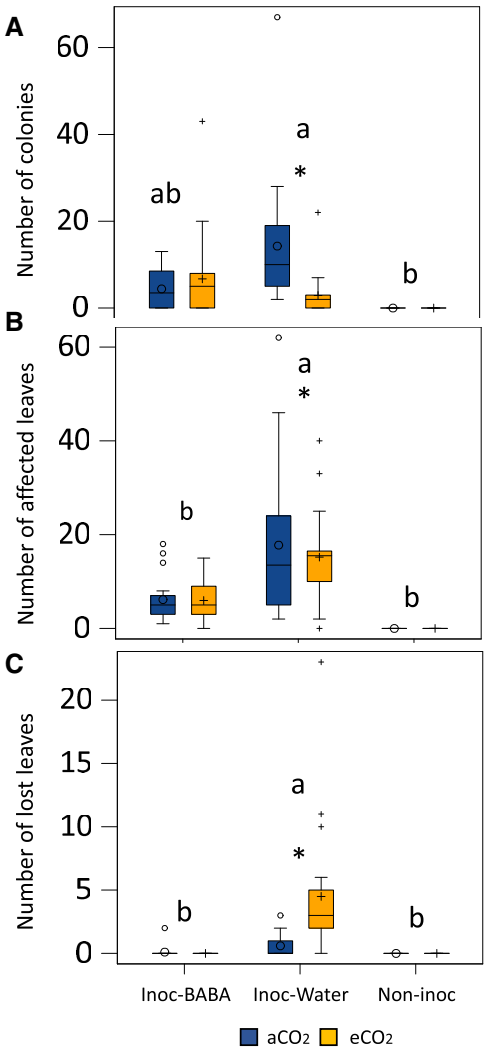

Figure 3. Combined effect of eCO_2 and BABA on PM disease.

Graphs indicate number of colonies (**A**), number of affected leaves due to the infection (i.e. presenting colonies) (**B**) and number of lost leaves at the end of the experiment (14 dpi) (**C**). Lowercase letters inside the figures represent statistically significant differences between groups: Inoc-BABA representing infected seedlings treated with BABA; Inoc-water representing infected seedlings and Non-inoc representing non-infected seedlings (Tukey post-hoc test; $P < 0.05$; $n = 8$ for inoculated plants and $n = 4$ for non-inoculated plants). Asterisks indicate statistical differences between CO_2 concentration (Tukey post-hoc test; $P < 0.05$; $n = 8$ for inoculated plants and $n = 4$ for non-inoculated plants).

inoculated plants (Figure 5D), however no differences were found in BABA-treated plants. Interestingly, whereas DRGR values were reduced in all treatments as the experiment progressed, the values of non-inoculated plants increased under eCO_2. (Figure 5D).

At the final point right after measuring T3, destructive measurements in root length and root and shoot biomass were recorded. BABA-inoculated seedlings presented higher root length values than the non-inoculated and water-inoculated seedlings (Figure 6A) and this observation was more pronounced under aCO_2. eCO_2 resulted in shorter roots in both inoculated water- and BABA-treated seedlings (Figure 6A) however no differences were found in the non-inoculated plants. Similar patterns were observed in root biomass than in root length as BABA treated plants had heavier roots systems (Figure 6B). Interestingly, however, no significant differences in root biomass were found between CO_2 concentrations. Shoot biomass revealed no statistically significant differences between aCO_2 and eCO_2 or between different treatments (Figure 6C).

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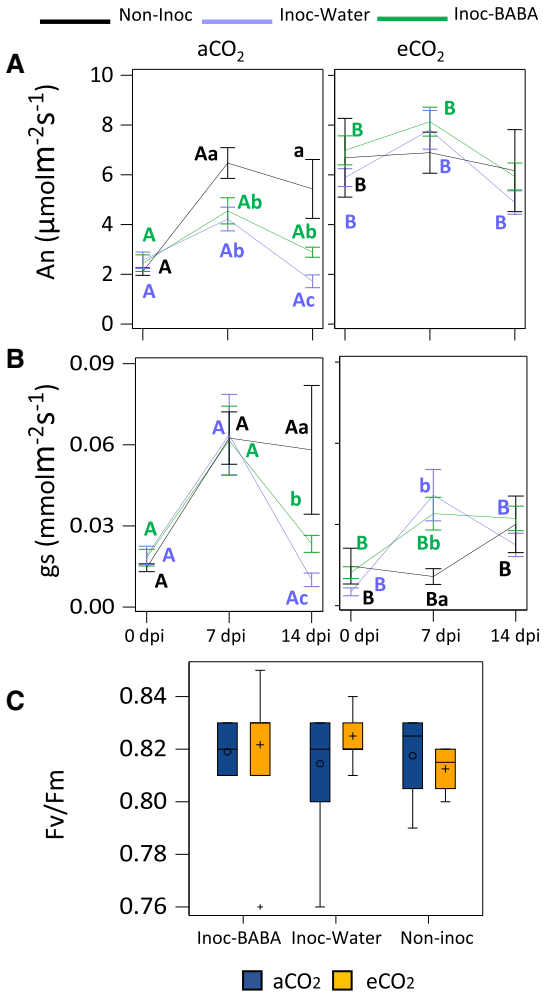

Figure 4. Combined effect of elevated CO_2 levels and BABA treatment on physiological parameters.
Net photosynthetic rates (An) (**A**), Stomatal conductance (Gs) (**B**) and Photosynthetic Efficiency of Photosystem II (**C**). The horizontal axis represents the measurement at 0, 7 and 14 dpi. Lowercase letters inside the figures represent statistically significant differences between groups: Inoc-BABA (green) representing infected seedlings treated with BABA; Inoc-water (light blue) representing infected seedlings and Non-inoc (black) representing non-infected seedlings (Tukey post-hoc test; $P < 0.05$; $n = 8$ for inoculated plants and $n = 4$ for non-inoculated plants). Capital letters indicate statistical differences between CO_2 concentration (Tukey post-hoc test; $P < 0.05$; $n = 8$ for inoculated plants and $n = 4$ for non-inoculated plants).

Discussion

Here, we have attempted to disentangle growth and defence phenotypes caused by elevated CO_2 (eCO_2). Even when it could be considered as a plant growth stimulant (e.g. increased stem growth, woody biomass, leaf area index and root number), eCO_2 has been demonstrated to trigger a stress response in plants [55, 56] and shown to affect the plant's defence capacity, although in the latter, highly contrasting phenotypes have been observed [46, 48, 57]. For instance, previous studies on the direct defence mechanisms affected by eCO_2 have demonstrated that enhanced susceptibility is based on the observations that eCO_2 alters the plant concentrations of salicylic acid (SA), jasmonic acid (JA) and/or polyphenols [5, 58, 59]. In contrast, in Arabidopsis, eCO_2 has been shown to affect cell-wall defence, through the enhanced deposition of callose upon insect attack [60, 61], the up-regulation of a key gene involved in callose biosynthesis [62] and the increased production of starch [41], a key regulator of callose [63, 64]. These results suggest that eCO_2 triggers enhanced callose deposition, which is correlated with a phenotype of enhanced resistance. Therefore, the up or down-regulation of specific defence mechanisms could affect the contrasting resistance and susceptibility phenotypes triggered by eCO_2 in

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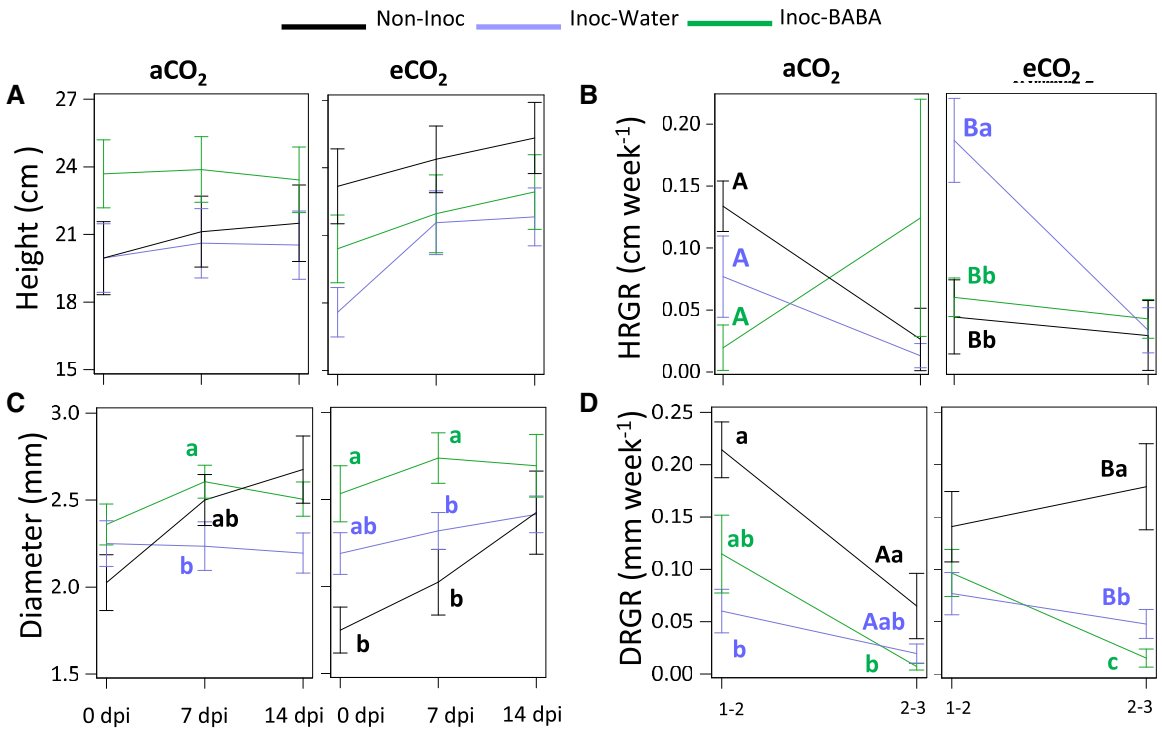

Figure 5. Combined effect of eCO_2 levels and BABA treatment on morphological parameters.

Plant height (**A**), height relative growth rate (HRGR) (**B**), trunk diameter (**C**) and trunk relative growth rate (DRGR) (**D**). The horizontal axis represents the measurement at 0, 7 and 14 dpi. Lowercase letters inside the figures represent statistically significant differences between groups: Inoc-BABA (green) representing infected seedlings treated with BABA; Inoc-water (light blue) representing infected seedlings and Non-inoc (black) representing non-infected seedlings (Tukey post-hoc test; $P < 0.05$; $n = 8$ for inoculated plants and $n = 4$ for non-inoculated plants). Capital letters indicate statistical differences between CO_2 concentration (Tukey post-hoc test; $P < 0.05$; $n = 8$ for inoculated plants and $n = 4$ for non-inoculated plants).

herbaceous plants. In oak seedlings, we have identified that worryingly, eCO_2 results in enhanced susceptibility to the already damaging pathogen causing PM disease (Figure 1B–D, 3 and Supplementary Figure S2). This is in line with the results of Lake and Wade [46] and Khan and Rizvi [58] as they observed the increased susceptibility to cucurbit PM *Erysiphe cichoracearum* under eCO_2 (800 ppm and ~600 ppm, respectively). This increased susceptibility was associated with greater fungi colonisation, changes in stomatal abundance and foliar thickness. Similarly, Teng et al. [65] described reduced stomatal density and stomatal index of leaves under 700 ppm associated with susceptible phenotypes. Therefore, further research in stomata abundance in oak seedlings would provide answers on the mechanisms by which eCO_2 exposure results in enhanced susceptibility. In agreement with this susceptible phenotype, other researchers have also shown this effect against PMs, however, these studies demonstrated that enhanced susceptibility only occurred when eCO_2-exposed plants were also grown under high temperatures [48, 50]. Considering that global temperatures are expected to increase considerably, that temperature is a determinant factor in the virulence of PM-causing pathogens [7], and our results described here on the effect of eCO_2, it is likely that the added stress of high temperatures could result in an even further pronounced susceptibility phenotype. Therefore, future research should focus on taking into consideration the impact of climate change associated factors alongside the effect of eCO_2.

The exploitation of the plant immune system through induced resistance (IR) has been proposed as an alternative for plant disease control. The IR compound β-aminobutyric acid (BABA) provides protection in a variety of plants against PM diseases [24, 30, 31, 66]. BABA enables defences by priming callose deposition and the activation of the SA-dependent defence pathways [24, 25, 33, 66]. The mechanisms by which BABA-IR manifests in oak seedlings against this biotrophic pathogen are currently under study, however we can hypothesise that as described in other species, BABA will induce resistance through priming of SA and callose [67], which are known to be highly effective against PMs [68, 69]. Whereas eCO_2 resulted in enhanced susceptibility,

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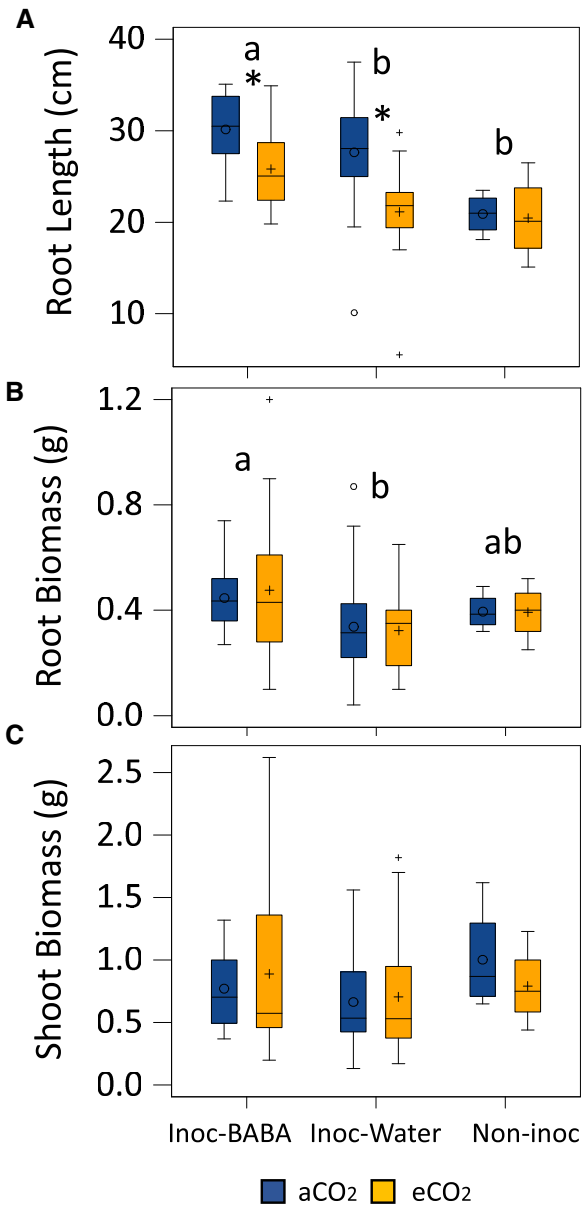

Figure 6. Combined effect of eCO_2 levels and BABA treatment on root traits.

Graphs represent root length (**A**), root biomass (**B**) and shoot biomass (**C**) at the end of the experiment (14 dpi). Lowercase letters inside the figures represent statistically significant differences between groups: Inoc-BABA (green) representing infected seedlings treated with BABA; Inoc-water (light blue) representing infected seedlings and Non-inoc (black) representing non-infected seedlings (Tukey post-hoc test; $P < 0.05$; $n = 8$ for inoculated plants and $n = 4$ for non-inoculated plants). Asterisks indicate statistical differences between CO_2 concentration (Tukey post-hoc test; $P < 0.05$; $n = 8$ for inoculated plants and $n = 4$ for non-inoculated plants).

BABA-IR was not affected under eCO_2 conditions. Considering that eCO_2 has been shown to trigger an accumulation of SA and to promote cell-wall defence through the enhanced deposition of callose [60, 61, 70], it is easy to speculate that priming of these defence mechanisms remains intact under eCO_2. Further investigations are needed to unravel the effect of eCO_2 in IR and priming of oak seedlings.

Enhanced photosynthesis by eCO_2 has been described in multiple studies [36, 55, 65]. We have found that eCO_2 increases photosynthesis rates at the same time that reduces stomatal conductance, two key physiological parameters that influence plant growth, development and biomass production [65]. According to our results, in

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aCO_2 conditions, stomata were strongly closed at 0 dpi and that is why the conductivity and An are so low. Plants grown at eCO_2 showed an even lower conductivity while at the same time showing higher An. These observations could be as result of the CO_2 concentration in the chloroplast and intercellular spaces being very high thus saturating cells with CO_2, which could therefore allow plants to keep their stomata closed whilst increasing photosynthesis. This would consequently result in a higher water use efficiency, something that we observe in our experiments (Figure 4A,B). In addition, we did not observe statistically significant changes in photosynthetic efficiency of PSII (Fv/Fm) between treatments (Figure 4C). Considering that reduced Fv/Fm has been associated with the known induced stress of eCO_2 [51, 71, 72], our results indicate that in oak seedlings, eCO_2 does not result in stress. This contrasting result to the one observed in many other plant species can be due to the concentration of BABA used in our experiments, which are not sufficient to trigger a direct activation on defence mechanisms and consequently costs in growth [41, 73]. Moreover, in general, increased concentrations of CO_2 trigger enhanced growth in plants [57]. Indeed, we have observed increases in HRGR and DRGR triggered by eCO_2 (Figure 5B,D). Therefore, our results are in agreement with the literature on the effect of eCO_2 in increased photosynthesis and growth, however these effects are not linked with eCO_2-induced stress.

Some studies have focussed on evaluating changes in physiological and growth parameters upon biotic attacks under eCO_2. For instance, infection with PM of barley plants showed that RGRs of shoot and root dry weight were higher in PM infected plants growing at eCO_2 in comparison with plants grown at aCO_2, attributing that faster growth to the increased An [42]. Similarly, we found higher HRGR growing under eCO_2 at early stages of infection and higher DRGR at later stages of infection (Figure 5B,D). This correlated with higher levels of An under eCO_2 throughout the experiment (Figure 4A). The enhanced growth patterns at different stages of infection allow us to speculate that eCO_2 accelerates developmental processes as, naturally, seedlings invest first in aerial growth towards leaf production and then the engrossment of trunks. Importantly, however, only in aCO_2 grown plants we did observe a reduction in An and stomatal conductance after infection. Moreover, the increase in DRGR was much more pronounced in non-inoculated plants growing under eCO_2 than in inoculated plants. This is likely due to (i) a reduced net assimilation rate in PM-infected plants through a source effect, i.e. PM is directly responsible for a decrease in photosynthesis like documented in other foliar diseases [74] or; (ii) to the disease itself, caused by an obligate biotroph, which can also reduce the net assimilation rate through a sink effect, i.e. PMs divert carbon fluxes from growing plant organs to themselves for their own growth [75, 76]. Nevertheless, no changes were observed in An and stomatal conductance in inoculated seedlings growing under eCO_2. Thus, eCO_2 exposure at the levels used in our experiments results in enhanced physiological parameters irrespective of the infection status. The understanding of these parameters as the disease fully develops could bring further insights into the effect of eCO_2 in the pathosystem. BABA-IR is known to be coupled to a growth reduction in herbaceous plants due to the binding of BABA to its plant receptor [32] or/and the relocation of resources from growth to defence [34]. Our results confirm that BABA enhances resistance in oak seedlings against PM however, no detrimental effects in growth were found (Figs. 2A, 5B). Moreover, BABA treatment had no significant impact on physiological parameters (Figure 4A,B) at early infection stages. This suggests that the impact of BABA in growth does not occur while the infection has not fully developed. Interestingly, however, we observed that after infection, plants treated with BABA had physiological and growth values resembling closer to the ones of non-inoculated plants than the water infected ones in almost all parameters (Figs. 3–6). We consider this to be a buffering effect of BABA on the impact of the infection in physiology. Importantly, this buffering effect was again more pronounced in plants grown under aCO_2 than the ones grown under eCO_2, thus showing the clear driver effect of eCO_2 in physiology and providing a potential explanation of the reduced effect of BABA-IR under eCO_2. A reason for why this is happening could be associated with an increased C:N ratio of plant biomass caused by exposure to eCO_2 [56, 77, 78]. Fertirrigated basil plants with NH4+ have been shown to have less severe downy mildew disease [79]. Importantly, studies on *Vicia* and *Medicago* have shown that BABA treatments result in an increased N levels in leaves [80]. Therefore, it is easy to hypothesise that BABA reduces C:N ratios in oak seedlings to trigger effects on resistance to foliar diseases, which could explain part of the BABA-protective role as well as the weaker effect of BABA under eCO_2.

At the end of the experiment, root and shoot traits were assessed and compared. We found no differences in shoot biomass under eCO_2 in any of the treatments, which could be because mycelium or cumulative spore biomass can represent up to 50% of infected leaf biomass [75]. Similar lack of differences on dry mass were also observed on soybean plants infected with PM and grown under eCO_2 [47]. In addition, our experiments found no differences in root biomass between aCO_2 and eCO_2. This has been previously described when

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comparing C3 and C4 plant species and where an increase in An did not correlate with higher biomass [81]. Interestingly, however, we observed a significant reduction in root length under eCO_2 with respect to aCO_2-grown seedlings after infection. Therefore, in the presence of an infection under eCO_2, roots were shorter, but they had similar dry weights. To explain this observation, we need to revise the process of carbon (C) allocation to maximise trade-offs between growth and defence [82]. CO_2 fixed by photosynthesis in chloroplasts has several possible fates, but a considerable part of it ends up as sucrose, which is consumed in respiration and growth or is stored as solutes in vacuoles. Sucrose excess as result of eCO_2 can be exudated through the roots as organic acid derivatives such as malic acid [83], which in turn could acidify the soil surrounding the plant under eCO_2 and damage the tissue thus resulting in shorter roots. Surprisingly, soil-drench BABA treatment produced longer and heavier roots than the water-treated controls (Figure 6). This result could be due to the biostimulant role of amino acids for yield and growth improvements [84–86]. This biostimulatory effect of amino acids is caused by the modulation of plant molecular and physiological processes [87], including direct effects on C and nitrogen (N) metabolism and N uptake [88–90]. However, even when eCO_2 also triggered a reduction in the root length in BABA-treated seedlings upon infection, this phenotype was less pronounced than in inoculated water-treated plants. Considering the demonstrated effects of eCO_2 on the levels of defence compounds (e.g. SA) [5, 57, 59] and the mechanisms of action of BABA in other plant species (e.g. priming of SA-dependent defences) [26, 29, 30, 66], we could speculate that there are trade-offs between growth and defence driven by eCO_2.

The molecular mechanisms underlying growth and defence trade-offs under eCO_2 remain to be elucidated. Nevertheless, our results have shown that whereas eCO_2 increases photosynthesis and growth, it also enhances susceptibility to PM but does not hinder the effect of BABA, a plausible method of control of this disease in tree nurseries. Therefore, considering that oak trees are keystone trees of our European forests and that their regeneration is threatened by the high susceptibility of oak seedlings to PM disease, our results warrant further investigations on the risks of climate change associated with enhanced levels of atmospheric CO_2.

Materials and methods

Plant material and growth conditions

Acorns of *Quercus robur* 403 UK provenance [91] were germinated according to existing protocols [92, 93]. After 72 h, germinated acorns were transferred into individual root trainers (Maxi Rootrainers, Haxnicks, RT230101) containing 400 ml of Scott's Levington M3 Advance Pot & Bedding soil. Germinated acorns were transferred into the growth chambers (CONVIRON, Controlled Environments, Inc., Winnipeg, Canada) and grown at 16/8 h light day/night (600 µmol photons m^{-2} s^{-1}), 20°C/17°C cycle, 42% HR and irrigated to field capacity throughout the experiment. Two different CO_2 concentrations were used: ∼400 ppm for ambient CO_2 (aCO_2) and ∼1000 ppm for elevated CO_2 (eCO_2) which was maintained by a CO_2 canister connected to the CONVIRON cabinet settings. Delivery of the eCO_2 at the specified concentration was done and monitored by the cabinet's automatic control system and this was set up following manufacturer's specifications. Experiments where germination and growth homogeneity were required, acorn germination of the eCO_2 seedlings was delayed for 7 days.

β-aminobutyric acid treatment

β-aminobutyric-acid (BABA) was purchased from Sigma (Catalogue number A44207). Treatment solutions were freshly prepared on the day of treatment. BABA treatments were performed entirely as previously described for other plant species [26]. Four-weeks old seedlings were soil-drenched with 40 ml per plant of a 50 mM BABA solution, resulting in a final concentration in the soil of 5 mM. BABA treatments were performed once at 7 days prior infection. Control treatment plants were treated with water. Before treatments, water and BABA plants were kept in different trays to avoid chemical contamination. After treatments, watering was done in equal amounts to maintain BABA concentrations and irrigation status stable among plants and treatments, as previously described [26, 30, 33].

Pathogen infection and disease scoring

Powdery mildew (PM) causal agent *E. alphitoides* was cultivated and maintained on oak seedlings. Spores were collected by shaking infected leaves in water and 0.05% Silwet-L77 (CAS 27306-78-1, De-Sangrosse). Inoculations were performed by spraying inoculum containing between 1.5 and 1.7×10^6 spores/ml onto leaves

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of the entire oak seedlings until run off. After inoculation, plants were covered with plastic bags to maintain high relative humidity and placed back into the growth chambers. To allow for CO_2 diffusion inside the bags, perforations with needles were performed. Non-inoculated controls were sprayed with a solution of water and 0.05% Silwet-L77 and covered with plastic bags as inoculated plants. Similarly, to ensure the eCO_2 conditions inside the bags, micro-perforations with needles were performed. Disease was scored by counting the number of colonies per leaf, the number of leaves affected (i.e. presenting colonies) and the number of discarded leaves ('lost'), the latter parameter assessed by counting the number of leaf nodes without leaves. Three independent experiments were performed.

Leaf physiological measurements

Physiological measurements were conducted with a portable open gas exchange system (LI-6400XT, LI-COR, Lincoln, NE, U.S.A.) at three time points representing different infection stages: timepoint 1 (T1) at 0 days post infection (dpi), before infection — no symptoms; timepoint 2 (T2) at 7 dpi; and timepoint 3 (T3) at 14 dpi. Measurements were done consistently on the same leaf, which had been produced on the first leaf flush. Photosynthetic rate (An) and stomatal conductance (gs) were measured at a saturating light intensity of 700 μmol photons m^{-2} s^{-1} (as determined by light response curves of An) and a flow rate of 300 μmol s^{-1} [94, 95]. Reference CO_2 was set at either 1000 μmol mol^{-1} eCO_2 or 400 μmol mol^{-1} for seedlings grown under elevated or ambient CO_2, respectively. Maximum photochemical efficiency of photosystem II (Fv/Fm) was measured at the final experimental point (14 dpi) using a portable Pocket PEA chlorophyll fluorometer (Hansatech Instruments Ltd) in the same leaves used for gas exchange measurements after a 30 min dark-adaptation period [96].

Aerial plant growth parameters

Height and main stem diameter were used to assess growth. Height was determined with a ruler (30 cm, Helix L16) by measuring the length from the ground to the top of the main stem. Diameter was determined with a calliper (0.01 mm; Carbon Fiber Composite Digital Calliper, EAN 5421815474575) at a specific and consistent point of the main stem at the second node after cotyledons. Relative growth rates (RGRs) in height (HRGR) and main stem diameter (DRGR) were determined between T1 and T2 and T2 and T3, corresponding with 0 and 7 dpi and 7 and 14 dpi, respectively, using the formula:

$$\mathrm{RGR} = (\mathrm{InXt2} - \mathrm{InXt1})/(\mathrm{t2} - \mathrm{t1}),$$

where Xt2 and Xt1 represent seedling height (cm) or shoot diameter (mm) at time points t2 and t1 [97]. Three independent experiments were performed.

Root plant growth parameters

Plant material was collected at T3 (14 dpi) to perform destructive measurements. Root length was measured as the distance between the hypocotyl and tip of the primary root using a ruler (30 cm, Helix L16).

Biomass parameters

Biomass was calculated with the fresh and dry weight of roots and shoots at T3 (14 dpi). Material was collected in aluminium foil, weighted (fresh weight, FW), dried for two days in a 60°C oven until constant mass and weighed (dry weight, DW) with a laboratory scale (KERN EMB600-2).

Statistical analyses

Statistical and data analyses were done using PROC MIXED (SAS 9.4 Institute, Inc.) and Prims software (version 9, GraphPad Ld). Eight plants (four per CO_2 condition) were used for non-inoculated controls and this treatment was named 'Non-inoc'. Sixteen plants (8 per CO_2 condition) were used for the inoculated group treated with water and these were named 'Inoc-Water'. Similarly, 16 plants (8 per CO_2 condition) were used for the inoculated group treated with BABA and this treatment was named 'Inoc-BABA'. Analysis of Variance (ANOVA) was performed to test differences among treatments using threshold $P < 0.05$ for represented graphics at 14 dpi (end of experiment, and/or unique measurement). Repeated measurements ANOVA was used for treatments involving measurements at different time-points represented in time-course graphs. Tukey post-hoc

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test at $P < 0.05$ was used to compare means for all the ANOVA and repeated measurement ANOVA. For all analyses, residual plots and normality tests were generated to identify outliers and to confirm that variances were homogeneous and normally distributed, respectively. No outliers were removed from the data set.

Data availability

Physiological and morphological raw data are available in the GitHub repository via the link https://github.com/PlantPriming/Elevated-CO2-on-oak-defense.git for consult. The corresponding excel file contains different spreadsheets separating morphological, physiological data in time course and final point experiment (14 dpi) incorporating root traits values.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author contributions

E.L. conceived and designed the study and obtained core funding. R.S.L. design the experimental work pipeline. R.S.L., C.M., M.R. and M.A.M., conducted experiments and gathered data. R.S.L. and C.M. design the data analysis pipeline. C.M. and R.S.L. performed statistical analyses and data interpretation. R.S.L. and E.L. wrote the article with input from all authors.

CRediT Author Contribution

Estrella Luna: Conceptualization, Resources, Supervision, Funding acquisition, Methodology, Writing - original draft, Writing - review & editing. **Rosa Sanchez-Lucas**: Data curation, Formal analysis, Supervision, Investigation, Visualization, Methodology, Writing - original draft, Writing - review & editing. **Carolina Mayoral**: Conceptualization, Data curation, Formal analysis, Supervision, Writing - original draft, Writing - review & editing. **Mark Raw**: Methodology, Writing - review & editing. **Maria-Anna Mousouraki**: Formal analysis, Visualization, Methodology, Writing - review & editing.

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Abbreviations

BABA, β-aminobutyric acid; DRGR, diameter RGR; HRGR, height RGR; IR, induced resistance; PM, powdery mildew; RGRs, relative growth rates; SA, salicylic acid.

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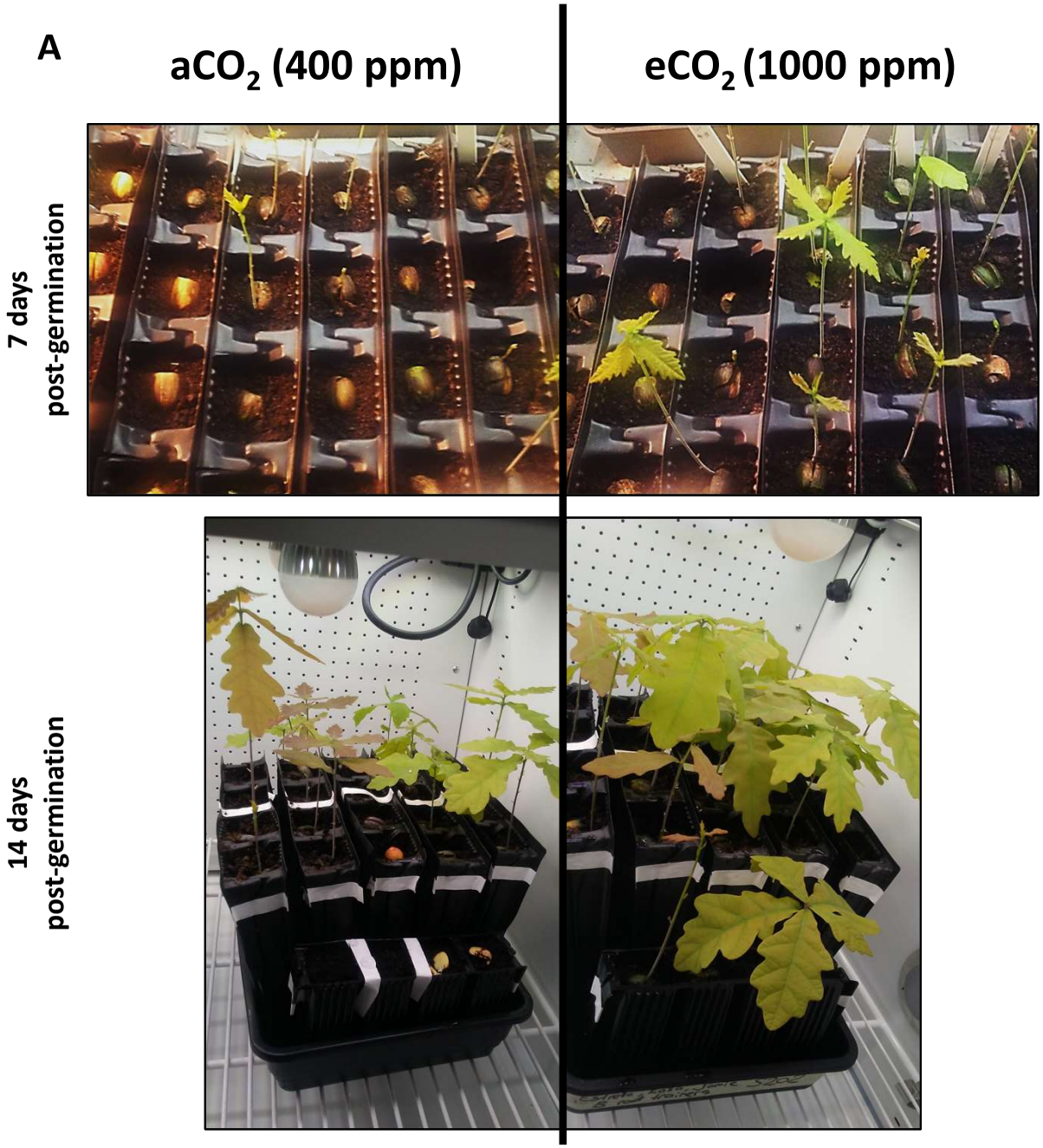
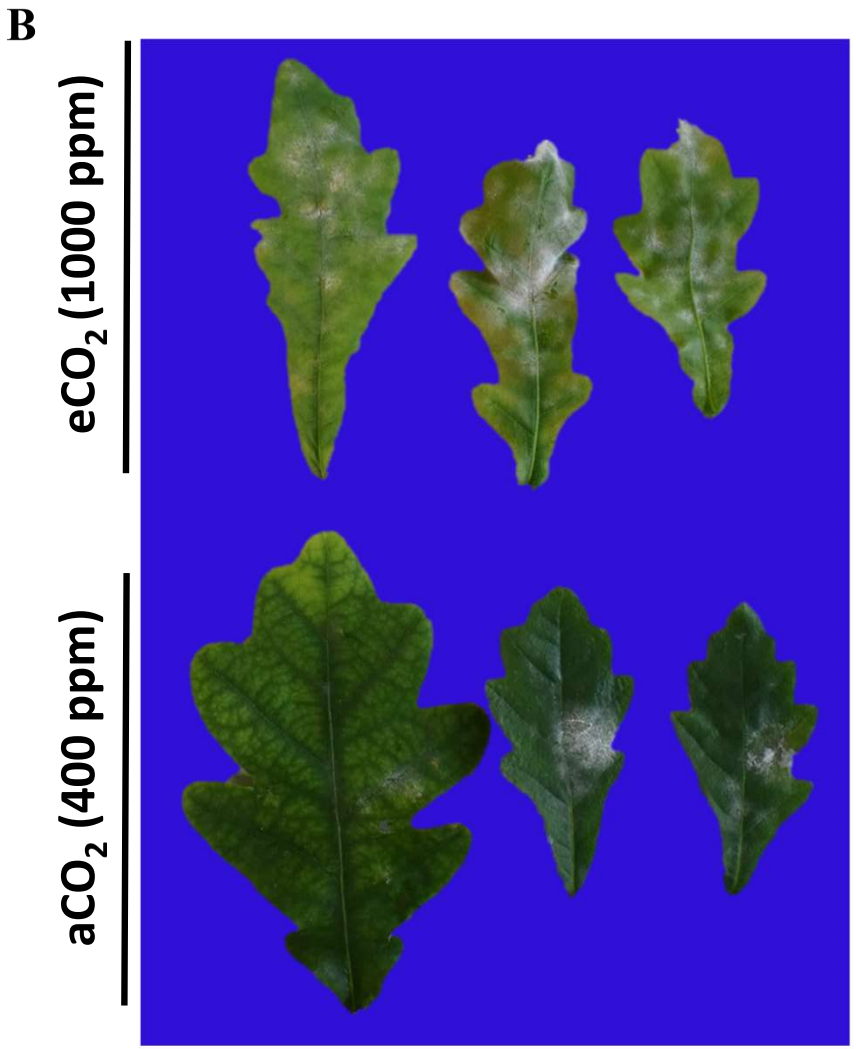
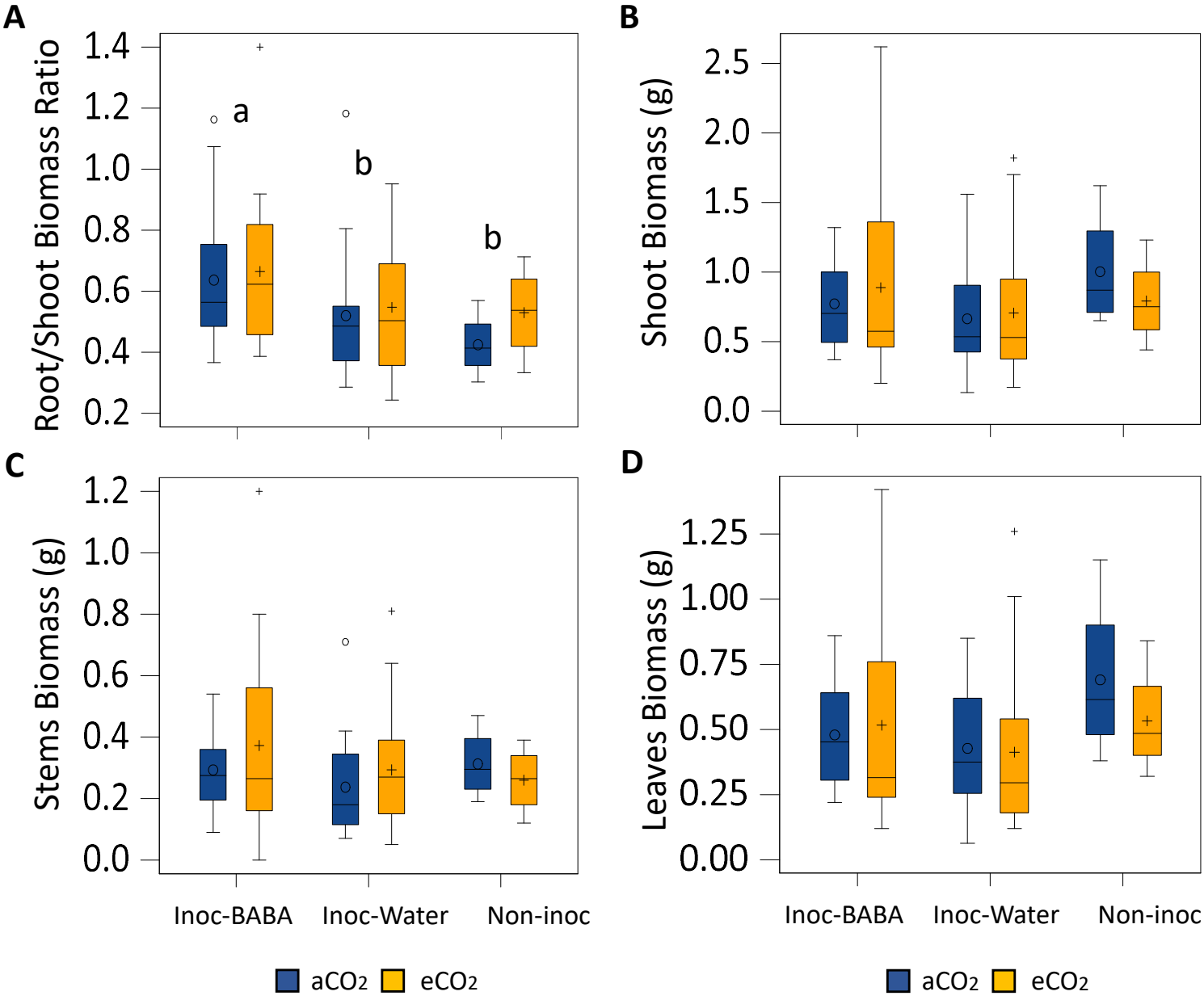
Supplem**entary Figure S1**. Visual effects of enhanced CO2 on A) growth at early timepoints and B) powdery mildew resistance in oak represented







Supplementary Figure S2. Combined effect of enhanced CO2 levels and BABA treatment on biomass allocation in roots/shoot ratio **(A)**, shoots **(B)**, stems **(C)** and leaves **(D)** at the end of the experiment. Lowercase letters inside the figures represent statistically significant differences between groups: Inoc-BABA representing infected seedlings treated with BABA; Inoc-water representing infected seedlings and Non-inoc representing non-infected seedlings (Tukey post-hoc test; $p<0.05$; n=8 for inoculated plants/n=4 for non-inoculated plants).

Supplementary Figure S3. Combined effect of enhanced CO2 and BABA treatment on oak seedlings phenotypes. Inoc-BABA represents infected seedlings treated with BABA; Inoc-Water represents infected seedlings treated with water and Non-inoc represents non-infected seedlings.