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# Impact of environmental conditions, stress severity and dose application on caffeine-related improved lentil productivity

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ABSTRACT

Flowering and pod formation are two of the most sensitive pheno-phases in lentil (*Lens culinaris* Medik.) development, and both can be strongly influenced by environmental conditions, biotic or abiotic stress. Caffeine (1,3,7-trimethylxanthine), a secondary metabolite within purine alkaloids, has a strong potential for its use as an active ingredient in biostimulant formulations. To evaluate the impact of environmental conditions, stress type, and dose on caffeine application effectiveness, we compared how various doses of caffeine influenced lentil production, pod formation, reproductive phenology, and foliar stress markers in irrigated plants, water-stressed plants, and plants exposed to the stem holoparasite field dodder (*Cuscuta campestris* Yunck.) in a controlled greenhouse experiment. Three caffeine concentrations ($10^{-5}$ M, $10^{-4}$ M, and $10^{-3}$ M) were tested, which were applied twice (at initial flowering and pod formation), and compared to a control treatment (without caffeine). Results showed that the highest caffeine dose increased lentil production by nearly 50% in irrigated plants, but not in water-stressed plants or those exposed to the holoparasite. Both water deficit and parasitism had a very negative impact on lentil production, with a yield reduction of 66% and 87% respectively, effects that were not counterbalanced by caffeine application. Furthermore, foliar stress markers, such as $F_v/F_m$ ratio and malondialdehyde content, showed a much stronger sensitivity of lentil plants to biotic than abiotic stress, and a higher impact of both stresses on flowering and fruit formation than on the vegetative physiological status. We conclude that (i) both abiotic and biotic factors strongly negatively influence the physiology of lentil plants, in particular pod and lentil production; and (ii) environmental conditions and both abiotic and biotic stresses have a strong impact on the potential response of plants to caffeine when used as a potential ingredient for biostimulant formulations.

## 1. Introduction

Several strategies have been developed to boost crop production in the actual highly demanding agricultural sector. Among them, a promising and environmental-friendly approach is the use of natural plant biostimulants with the aim of improving plant growth, flowering, fruit set, nutrient use efficiency, and ultimately crop productivity (Du Jardin, 2015). Alkaloids are small organic molecules, secondary metabolites of plants, which are derived from nitrogen metabolism (Ashihara and Crozier, 2001). These molecules are nitrogen stores, growth regulators, and are involved in the plant defense against herbivores and pathogens as well as in allelopathic activity, properties that make them a good choice as a plant biostimulant component (Fritsch et al., 2017). Caffeine (1,3,7-trimethylxanthine),a purine alkaloid, is well-known for its antimicrobial, fungicidal, anti-herbivoral, and allelopathic activity in plants (Ashihara and Crozier, 2001; Kim et al., 2015), and it might have some potential to mitigate stress through its modulation of purine metabolism, as shown in Arabidopsis (Watanabe et al., 2014; Nourimand and Todd, 2016). Indeed, Emanuil et al. (2022) showed that exogenous application of caffeine in spinach reduced cadmium toxicity stress and improved production. Nevertheless, the potential use of caffeine as an active ingredient for biostimulant formulations, and how environmental conditions, as well as biotic and abiotic stress, influence its effectiveness is insufficiently described. This is why it would be essential that caffeine

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could be studied not only to become a component for biostimulant products in order to promote the production and protection against abiotic stresses but also as a component of defence products facing abiotic stresses.Plants are usually exposed to a wide range of abiotic and biotic stresses caused by physicochemical (e.g., drought, heat, salinity) and living organisms (e.g., weeds, fungi, bacteria, nematodes). These stressors have a large impact on the phenological, morphophysiological, and biochemical functioning of plants and their metabolism (Parmesan and Yohe, 2003), which adversely affects their growth, development, and productivity (Hatfield et al., 2011; Nelson et al., 2014). Among all the abiotic stresses that affect plants, water deficit is often one of the most important limiting factors for crop production worldwide due to photosynthetic limitations, including photoinhibition of the photosynthetic apparatus (Dahal et al., 2014; Farooq et al., 2017). On the other hand, parasitic plants, such as *Orobanche* sp. and *Cuscuta* sp., are weeds considered an important threat and a biotic stress due to their negative impact on host plants and agricultural production (Bari et al., 2019). Both of them, abiotic and biotic stress factors can eventually cause several physiological alterations, including severe membrane lipid peroxidation, leading to cell death (Rajput et al., 2021).

Lentil (*Lens culinaris* Medik.) is a domesticated annual legume crop, which has been part of the human diet since the beginnings of agriculture. The actual global lentil production is around 6,54 million tonnes per year (FAOSTAT, 2020), and it is expected to increase in the coming years because of the growing demand for plant-based protein and environment-friendly agriculture, as it is a nitrogen-fixing crop that plays an important role in nitrogen cycling and can increase N availability for subsequent crops (Kirkegaard et al., 2008). Lentils are severely affected by drought events (Araújo et al., 2015) and weed infestation, especially holoparasitic plants such as *Cuscuta campestris* (Phogat et al., 2003; Muehlbauer et al., 2006). Since holoparasitic plants lack or have very limited photosynthesis capability, they obtain all their water, carbon, and nutrients from their hosts, causing not only a nutrient imbalance to the host but also a water deficit, resembling a drought event. Particularly, lentils can be highly sensitive to water deficit at different pheno-phases, including seedling, flowering, or pod formation stages (Mishra et al., 2014), reducing the economic yield by up to 70% (Farooq et al., 2017). Sehgal et al. (2017) described the effects of drought and heat on the growth and yield of *L. culinaris* during reproductive development, showing a severe yield loss due to abiotic stress. Nonetheless, very little is known about the holoparasitism response in this species, in particular the consequences of parasitism on seed production. Moreover, the effects of abiotic and biotic stresses in plant physiology and yield loss in *L. culinaris* have never been compared thus far.

We aimed here at exploring the potential use of caffeine individually as an active ingredient to improve yield, as a first step of considering its use in biostimulant formulations and/or defence products. For this purpose, we evaluated how environmental conditions, plant response to abiotic stress and biotic stress, and caffeine dose influenced yield, phenology, and foliar stress markers. We hypothesized that caffeine might exert a positive role on yield by mitigating stress on plants and that this effect may be more visible at the highest caffeine doses and under more severe stress conditions.

## 2. Material and methods

### 2.1. Plant material and growth conditions

Seventy-two pots with four lentil plants in each one (*Lens culinaris* cv. Pardina) were obtained from seeds, which were provided by Hort del Silenci S.A. (Eco Artesa 2058, Barcelona, Spain), and sown in pots of 1 L capacity containing a mixture of perlite:peat:vermiculite (2:1:1, by volume) in a constant environment chamber (12-h photoperiod, photosynthetically-active photon flux density [PPFD] of ~ 150 μmol $m^{-2}$ $s^{-1}$ provided by cool white fluorescent lamps, air temperature between 21 and 23ºC). After 4 weeks of growth, plants were arranged as a randomized complete block design in six replicates and grown in a greenhouse under long day to induce flowering with controlled photoperiod (16 h- photoperiod, which was provided by high pressure sodium lamps that supplied an additional PPFD of ~ 50 μmol $m^{-2}$ $s^{-1}$). Air temperature and relative humidity (HR) were continuously recorded throughout the experiment (Fig. 1) and the maximum daily PPFD received by plants was ~ 950 μmol $m^{-2}$ $s^{-1}$. Plants were always irrigated as per crop requirement with 50% Hoagland solution until the water deficit and parasitism treatments began on December 29th, 2021.

### 2.2. Caffeine applications and stress treatments

Plants were grouped into three different conditions, taking twenty-four individuals in each one. Water deficit and holoparasitism treatments were initiated on December 29th, 2021, when 50% of the plants reached flowering. The three conditions were (1) irrigated plants (watered with 50%-Hoagland solution three times a week), (2) water deficit (watered with a 50%-Hoagland solution once a week), and (3) holoparasitism (watered as irrigated plants but exposed to field dodder [*Cuscuta campestris* Yunck.] stem parasitism). Six days before stress initiation, on December 23th, 2021, *C. campestris* seeds were scarified by soaking in concentrated $H_2SO_4$ for 30 min to break seed dormancy, then washed with distilled water, and germinated in petri dishes containing two layers of wet filter paper with deionized water (dark conditions, air temperature between 21ºC and 23ºC). Four days after germination, seedlings (~5 cm length) were placed at the base of each lentil plant stem. Between December 29th and 30th, haustoria were formed. Stress initiation was considered then when the host-parasitic plant connection has just started to develop.

Caffeine treatments were applied twice to lentil plants; the first time coinciding with the onset of stress treatments (with 50% of plants at flowering stage) and the second time after one week (when most of the plants have initiated pod formation and filling) (Fig. 1). Three different caffeine concentrations were chosen starting from a hormonal concentration ($10^{-5}$ M),and increasing with two higher doses($10^{-4}$ M, and $10^{-3}$ M) and all were compared to a control (without caffeine). Treatments were applied by foliar spray to six individuals for each condition described, always two hours after sunset. Caffeine solutions were prepared by the dilution of pure caffeine into de-ionized water. All solutions contained 0.01% of Tween-20 as a surfactant. De-ionized water with 0.01% Tween-20 was sprayed as a control. The volume of sprayed solution was 50 ml per plant each time.

### 2.3. Phenology

Reproductive plant phenology was assessed three times a week from the start of treatments to assess the duration of each reproductive stage. Phenological observations were recorded at various stages, including (L1) first flower in any node, (L2) first pod formation in any node, (L3) initiation of pod filling, (L4) first fully pod in any node, (L5) at least one brown fully pod in any node, (L6) more than one brown pod and half of the plant with yellow leaves, and (L7) completely dried and dead plant.

### 2.4. Pod filling and seed production

As plants reached full maturity, seeds were harvested, and yield traits were recorded once a week. Number of filled and unfilled pods, filled and unfilled pods mass, total seed production (total number of lentils produced and their mass), and seed thickness, area, and moisture were measured. All parameters were estimated from every pot, where four lentil plants had been grown. Seed thickness and area were measured from five seeds of each lentil plant with a picture taken with white background and processed with ImageJ (1.52p, National Institutes of Health, Bethesda, MD, United States). To determine seed moisture, five fresh lentils were weighed, oven-dried for 3 days at 80ºC until constant

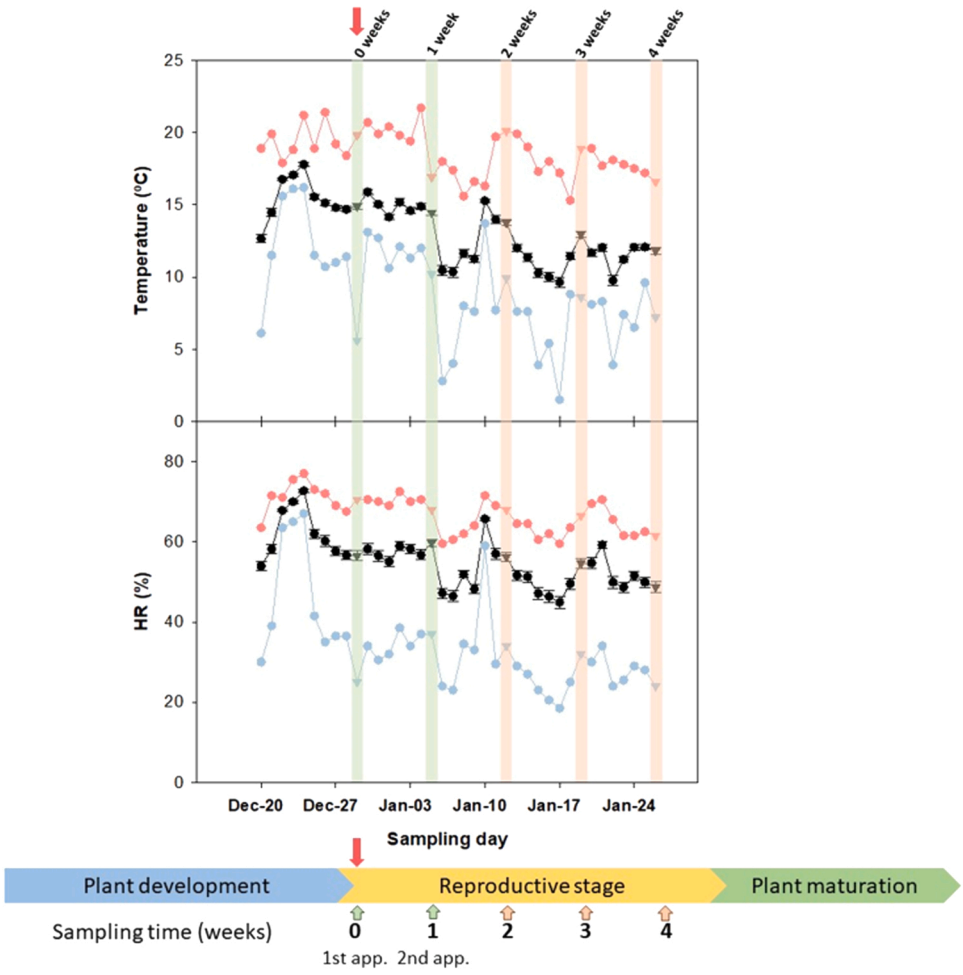


**Fig. 1.** Environmental conditions in the greenhouse, including air temperature and relative humidity (HR), during the experimental period. Black lines represent average daily values, while red and blue lines show maximum and minimum daily values, respectively. Green blurred lines depict application times, which coincided with the two initial sampling points, while orange blurred lines show the three subsequent sampling points. A red arrow indicates the onset of treatments (water deficit and parasitism).

weight, and weighed again. Seed moisture (%) was calculated by the difference between the fresh and dried lentil seed.

### 2.5. Foliar stress biomarkers

To evaluate the physiological status of leaves, leaf samples were collected at midday on 29th December (week 0), 5th January (week 1), 12th January (week 2), 19th January (week 3), and 26th January (week 4). In each sampling, six young, fully developed leaves from each lentil plant were sampled. One left-apical leaflet was used to determine the maximum efficiency of PSII ($F_v/F_m$ ratio), and the entire leaf was used to determine the water status, and the leaf mass per unit area (LMA). The other leaves were immediately frozen in liquid $N_2$ and stored at − 80ºC for subsequent malondialdehyde (MDA) analyses.

Relative water content (RWC) was calculated using the formula RWC= 100 x (FW-DW) / (TW-DW), where FW is the leaf fresh mass measured on sampling, TW is the turgid mass measured after rehydrating the leaves for 24 h in the dark at 4 °C, and DW is the dry mass measured after oven-drying the leaves at 80 °C until constant weight. Hydration (H) was calculated as (FW-DW)/DW. Furthermore, leaf area was measured with a flatbed scanner and processed with ImageJ (1.52p, National Institutes of Health, Bethesda, MD, United States). LMA was calculated as DW/ leaf area.

The maximum efficiency of PSII photochemistry ($F_v/F_m$ ratio), an indicator of photoinhibition of the photosynthetic apparatus, was measured in dark-adapted leaves for at least 1 h, using a portable chlorophyll fluorimeter (Mini-PAM II Photosynthesis Yield Analyser, Walz, Germany).

The extent of lipid peroxidation was determined by measuring a secondary lipid peroxidation product (malondialdehyde, MDA). To estimate MDA content, the thiobarbituric acid-reactive substances (TBARS) assay, a protocol that considers the potential influence of interfering compounds, was used (Hodges et al., 1999). 50 mg were extracted with 0.6 ml of ethanol 80% (v/v) containing 0.01% (w/v) BHT, using ultrasonication for 30 min and 15 min (Bransonic ultrasonic bath 2800, Emerson Industrial, Danbury, CT, United States), vortexing the extract before, between, and after ultrasonication. The extract was then centrifugated at room temperature for 13 min at 9000 rpm (Hettich Universal 32 R centrifuge, Thermo Fischer Scientific), the supernatant was recovered, and the pellet was re-extracted twice using the same procedure. The final extract volume was 1.8 ml. Then, for each extract one glass tube was used for the negative and one glass tube for the positive TBA reactions; (-) -TBA, with 500 µl extract + 500 µl 20% trichloroacetic acid (w/v) with 0.01% BHT (w/v), and (+) +TBA, with 500 µl extract + 500 µl 20% trichloroacetic acid (w/v), 0.01% BHT (w/v) and 0.65% thiobarbituric acid (w/v). Both positive and negative tubes were oven-incubated for 25 min at 95ºC. The reaction was stopped by moving the tubes to the fridge at 4ºC for 10 min. After centrifugation at 9000 rpm at room temperature for 7 min (Hettich Universal 32 R centrifuge, Thermo Fischer Scientific), the MDA content was analysed by spectrophotometry by reading absorbances at 440, 532, and 600 nm, and then quantified following the equations described by Hodges et al. (1999).

### 2.6. Statistical analysis

Statistical analyses were performed by one- or two-way ANOVAs, as indicated in figure legends. The Tukey test was used as a post-hoc

method. Differences were considered significant when *P* values were under the significance level $\alpha = 0.05$. Differences were also indicated in the text when they were marginally significant ($\alpha = 0.05$–$0.10$). All statistical tests were performed with RStudio (Boston, MA, United States).

## 3. Results

### 3.1. Caffeine treatments improved fruit and lentil production in irrigated plants only

Pod and lentil production, given as the total number of full pods and lentils produced per plant, as well as the total biomass of fruits and lentils produced per plant, decreased significantly in plants exposed to water deficit and holoparasitism compared to irrigated plants (Fig. 2). This reduction was even more drastic in plants exposed to field dodder infection than to water deficit (ANOVA, $P < 0.05$), with a yield reduction of 66% and 87% in terms of the number of lentils produced per plant under water deficit and holoparasitism, respectively, compared to irrigated plants. Caffeine application increased pod and lentil production in irrigated plants treated with the highest concentration ($10^{-3}$ M), showing an increase of 50% and 46% in the number of full pods and lentils per plant, respectively, compared to non-treated (control) plants, showing a dose-response effect. It should be noted that the effects of the highest caffeine dose differed from the other treatments in the number and biomass of full pods and lentils produced per plant, but the intermediate dose did not differ with any of them, neither from the control treatment nor from the highest dose, with average values between them (Fig. 2). Lentil biomass produced per plant increased as well at the highest caffeine concentration compared to the control, but effects were marginally significant only (ANOVA, $P = 0.09$). No significant differences among caffeine treatments were observed when plants were subjected to severe stress, either abiotic (water deficit) or biotic (holoparasitism). Lentil morphometry was not affected by

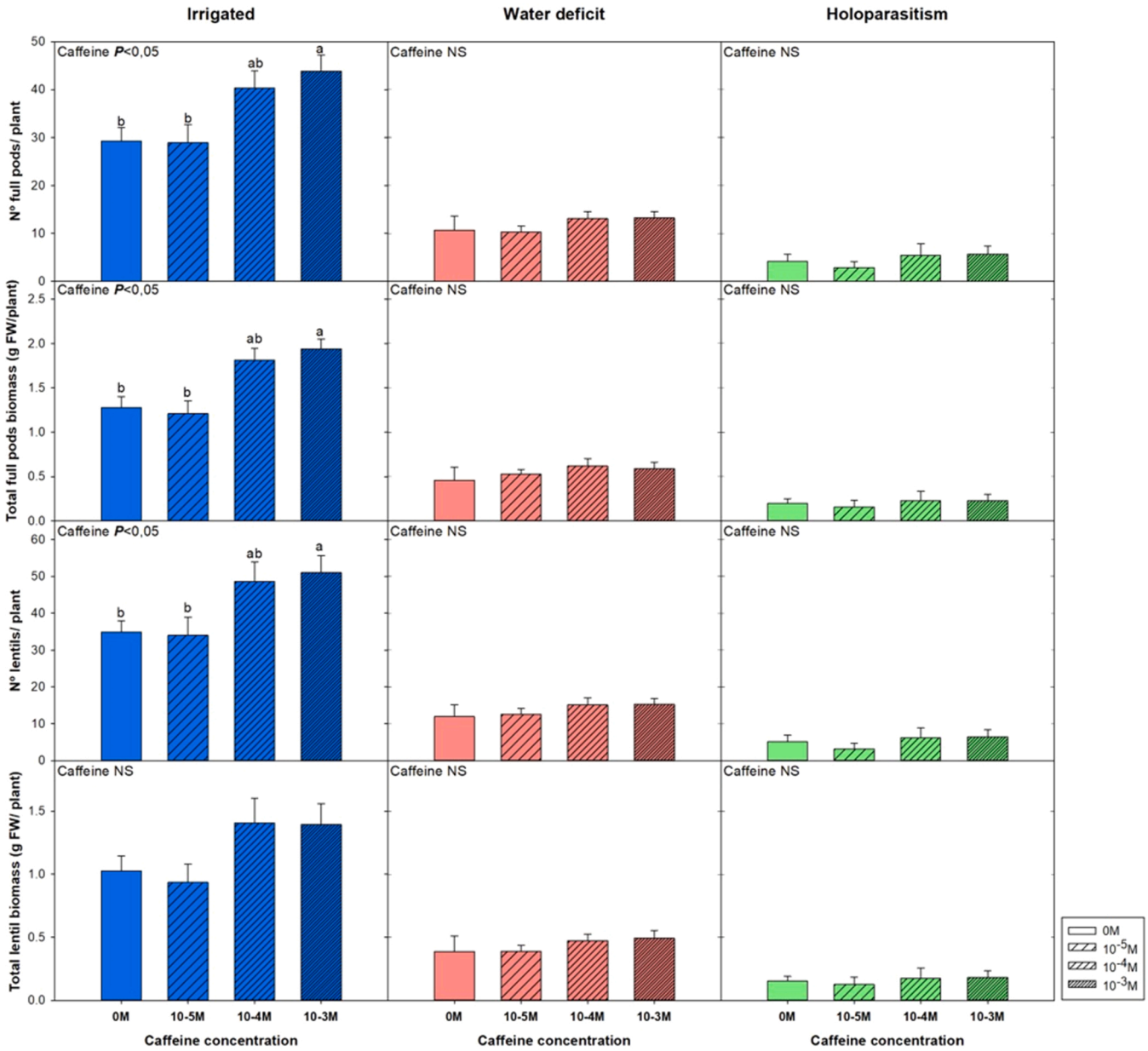


**Fig. 2.** Influence of caffeine treatments on productivity of lentil plants subjected to different conditions, including irrigated plants (blue), plants exposed to water deficit (red) and plants exposed to holoparasitism (green). Data show the mean ± standard error of n = 6 pots, using four plant sub-samples for each pot. Different letters indicate significant differences among caffeine concentrations according to Tuckey's post-hoc test ($P < 0.05$).

caffeine treatments under any of the conditions studied (irrigated plants, plants exposed to water deficit, and holoparasitism), showing no differences among treatments in lentil volume, area, or thickness (Suppl. Fig. 1).

### 3.2. Reproductive phenology was affected by water deficit and holoparasitism, but not by caffeine treatments

Reproductive phenology was not altered by caffeine treatments under any of the conditions studied (Fig. 3). Holoparasitism accelerated fruit maturity and plant senescence from pre-maturity stage (L5) to death. Lentil plants infested with field dodder achieved full maturity earlier than irrigated and water-stressed plants and water-stressed plants elapsed more time from plant maturity to death (L7) than irrigated plants or those exposed to biotic stress (two-way ANOVA, $P < 0.05$). Different growth conditions also affected the number of aborted pods. Plants exposed to holoparasitism produced fewer unfilled or aborted pods than water-stressed and irrigated plants (Suppl. Fig. 2). Nevertheless, the percentage of unfilled pods was similar between both stress conditions compared to irrigated plants, and no significant differences were observed in pod aborption when plants were treated with caffeine compared to controls (one-way ANOVA, $P > 0.10$).

### 3.3. Foliar stress biomarkers were mostly affected by water deficit and holoparasitism, but not (or very slightly only) by caffeine treatments

Although caffeine treatment at $10^{-3}$ M improved pod production and yield in irrigated plants, foliar stress biomarkers did not reveal any consistent improvement in leaf water contents (Fig. 4), leaf morphology (Suppl. Fig. 3), and indicators of photoinhibition ($F_v/F_m$ ratio) or membrane lipid peroxidation (MDA) in leaves (Fig. 5) due to caffeine treatments. The only difference caused by applying caffeine in irrigated plants was observed for the $F_v/F_m$ ratio, which was slightly reduced by caffeine concentrations of $10^{-4}$ M and $10^{-5}$ M in the last sampling point. However, it should be noted that these differences were quantitatively very small, they were observed in sampling 4 only, and the $F_v/F_m$ ratio was kept always at or very close to 0.80 (Fig. 5).

Caffeine application neither influenced any of the studied parameters in leaves of plants exposed to water deficit or holoparasitism, except for a slight improvement in leaf hydration at caffeine concentrations of $10^{-4}$ M during the last sampling point in plants exposed to holoparasitism (Fig. 4). Furthermore, results of two-way ANOVA revealed a significant effect for caffeine on lipid peroxidation, as indicated by MDA contents, in plants exposed to field dodder, which appeared to be associated to caffeine concentrations of $10^{-4}$ M and $10^{-5}$ M at samplings 1 and 4, but differences could not be confirmed by post-hoc analyses (Fig. 5).

Holoparasitism and water deficit accelerated leaf water loss (with

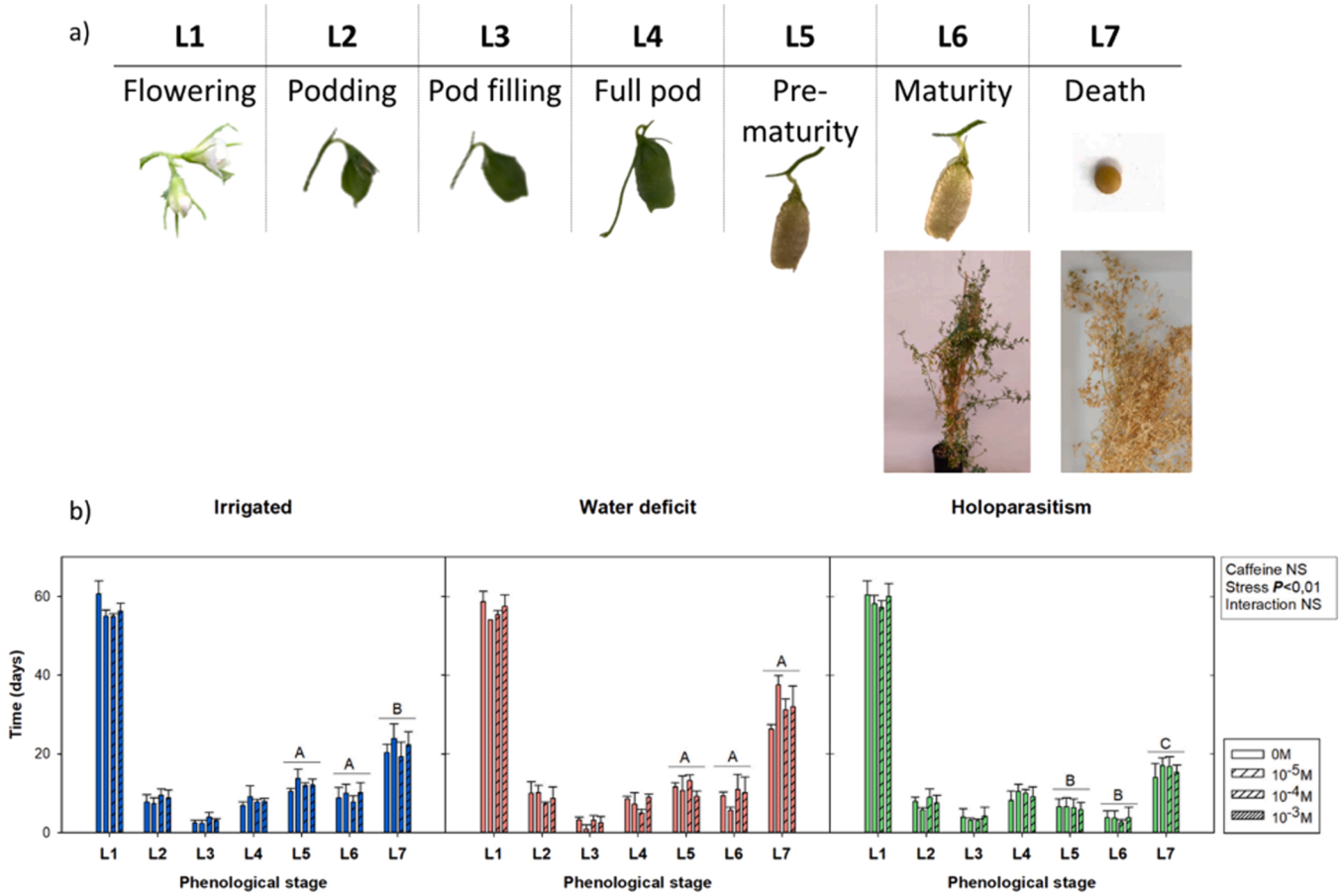


**Fig. 3.** Influence of caffeine treatments on reproductive phenology progression of lentil plants subjected to different conditions, including irrigated plants (blue), plants exposed to water deficit (red) and plants exposed to holoparasitism (green). **a)** Stages are defined as follows: (L1 or flowering) first flower in any node, (L2 or podding) first pod formation in any node, (L3 or pod filling) initiation of pod filling, (L4 or full pod) first fully pod in any node, (L5 or pre-maturity) at least one brown fully pod in any node, (L6 or maturity) more than one brown pod and half of the plant with yellow leaves, and (L7 or death) completely dried and dead plant. **b)** Time elapsed between stages. Data show the mean ± standard error of n = 6 pots, using four plant sub-samples for each pot. No significant differences were found among caffeine treatments in any condition (ANOVA, $P > 0.10$). Different capital letters indicate significant differences among stress conditions according to Tuckey's post-hoc test ($P < 0.05$).

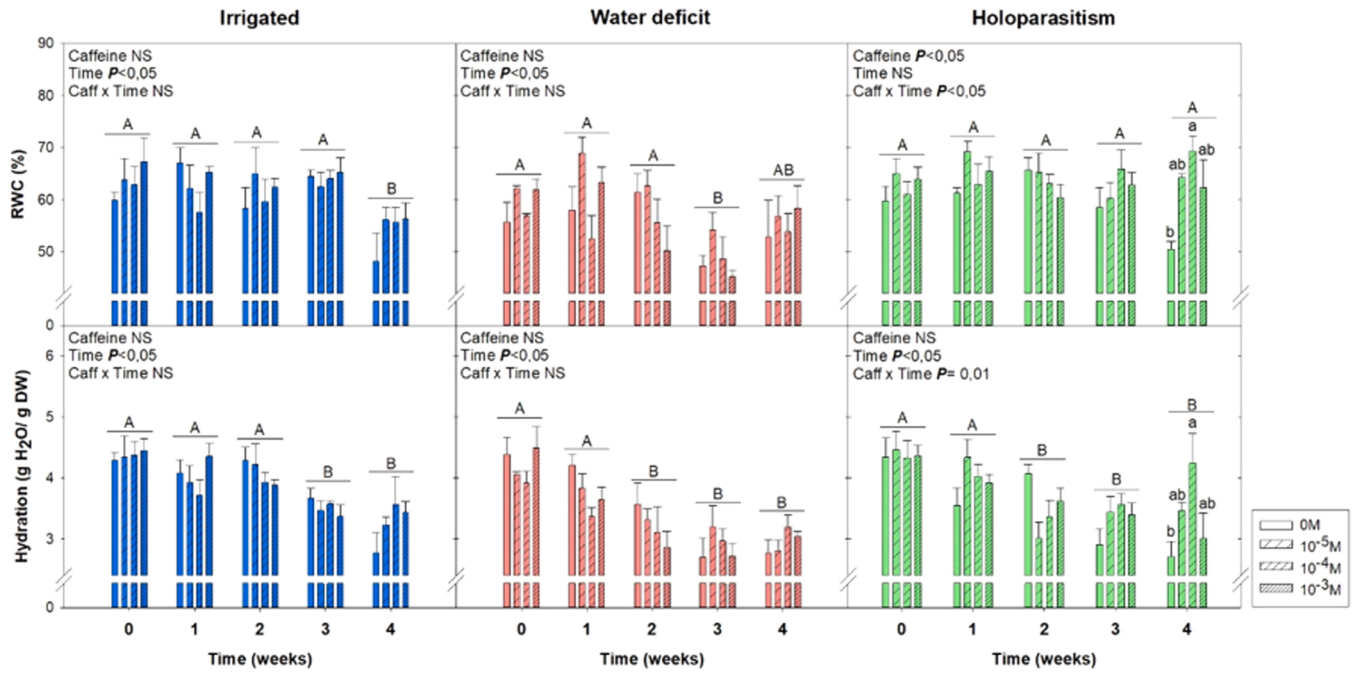


**Fig. 4.** Influence of caffeine treatments on leaf water content, including relative leaf water content (RWC) and leaf hydration (H), of lentil plants subjected to different conditions, including irrigated plants (blue), plants exposed to water deficit (red) and plants exposed to holoparasitism (green). Data show the mean ± standard error of n = 6 pots, using four plant sub-samples for each pot. Results of two-way ANOVAs are shown in the inserts. Different capital letters indicate significant differences between sampling points and small letters indicate differences among caffeine concentrations according to Tuckey's post-hoc test ($P < 0.05$). NS, not significant.

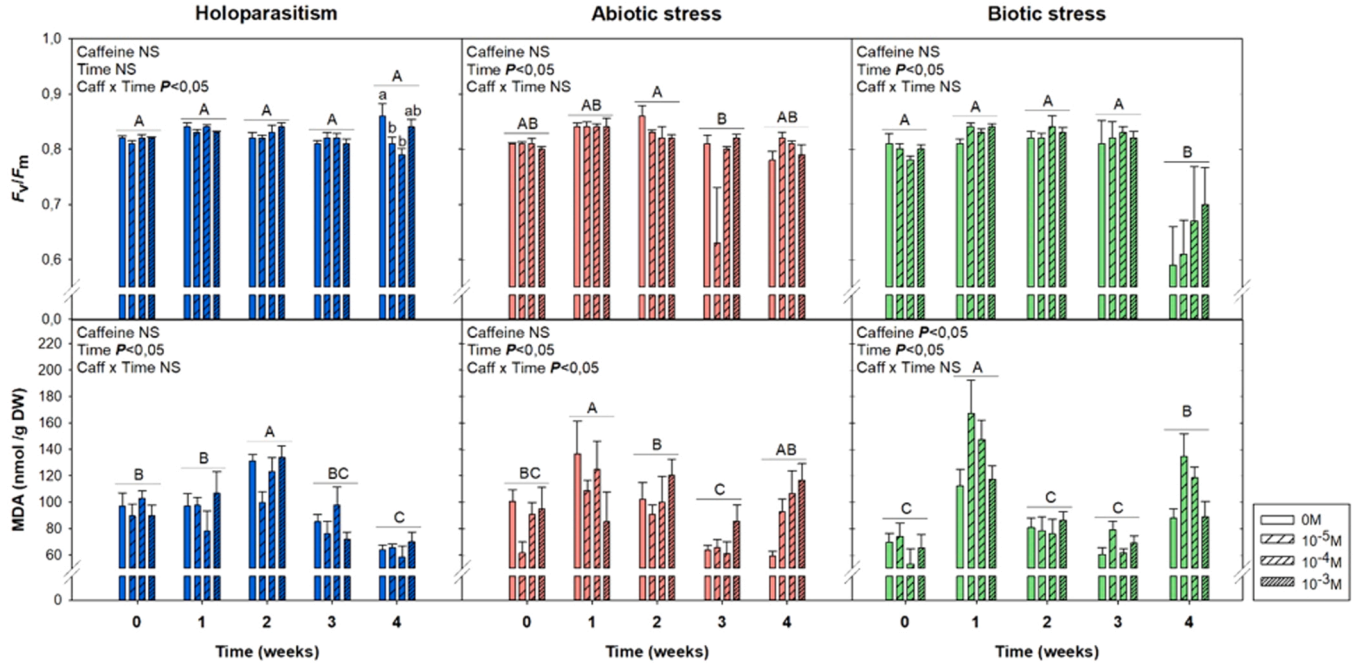


**Fig. 5.** Influence of caffeine treatments on the maximum efficiency of PSII photochemistry (*Fv/Fm* ratio), and indicator of photoinhibition to the photosynthetic apparatus, and malondialdehyde (MDA) contents, an indicator of the extent of membrane lipid peroxidation, in leaves of lentil plants subjected to different conditions, including irrigated plants (blue), plants exposed to water deficit (red) and plants exposed to holoparasitism (green). Data show the mean ± standard error of n = 6 pots, using four plant sub-samples for each pot. Results of two-way ANOVAs are shown in the inserts. Different capital letters indicate significant differences between sampling points and small letters indicate differences among caffeine concentrations according to Tuckey's post-hoc test ($P < 0.05$). NS, not significant.

reductions in leaf hydration appearing earlier in development than in irrigated plants, Fig. 4), promoted lipid peroxidation (particularly during pod formation [sampling 1] and senescence [sampling 4]), as well as photoinhibition, as indicated by reductions in the $F_v/F_m$ ratio, at the latest stages of development (Fig. 5). The extent of lipid peroxidation, estimated as MDA contents, increased at the first week of biotic and abiotic stress. This increase was higher in plants stressed by parasitism, coinciding with the first stages of field dodder development and

haustorium formation along the host stem. Then, it was reduced until the fourth week when it increased in both severe stress conditions, especially in the biotic stressed plants, at the same time as the reduction in the $F_v/F_m$ ratio. MDA content also increased in irrigated plants in the second week (Fig. 5), corresponding to a temperature and air humidity peak (Fig. 1). New leaves formed during the reproductive phase were thicker and smaller as plants became more physiologically mature, as indicated by increases in the leaf mass per unit area (LMA) in irrigated plants. LMA increased further in water-stressed plants, mainly due to a reduction in leaf area (Suppl. Fig. 3).

## 4. Discussion

Cool-season legumes such as lentil (*L. culinaris*) need low temperatures at the time of vegetative growth, while maturity requires warm temperatures, and the best temperature for its optimum growth has been found to be 18–30 °C (Sinsawat et al., 2004; Roy et al., 2012; Sita et al., 2017). Any significant alteration in climate leading to lentil growth, and more particularly reproductive development, outside this optimal temperature window, makes lentil production to be substantially reduced. Therefore, the use of biostimulants may be considered a good approach to improve the productivity of this very sensitive crop under fluctuating suboptimal conditions, and/or several abiotic and biotic stresses. In the present study, results show that the application of caffeine at a high concentration ($10^{-3}$ M) could help improve lentil production under fluctuating and non-optimal temperature conditions during plant reproduction. Nevertheless, when plants are stressed with severe abiotic or biotic stresses, the impact of caffeine is negligible. Furthermore, we have shown the serious impact of both abiotic and biotic stresses during the reproductive development stage of *L. culinaris*, not only on plant reproduction, fruit, and seed yield but also on the overall plant physiology and phenology.

Plant biostimulants are considered to be a potential tool to help mitigate economic losses associated with the negative impact that suboptimal growth conditions and stressors can cause to crops, by stimulating plants' natural nutrition process. Alkaloids are nitrogen-containing secondary metabolites that perform several functions such as plant defense against many parasites, as well as allelopathic activity. Caffeine is a purine alkaloid well-known for its application as a dietary component. The caffeine content in many beverages and food products leads to the presence of this molecule in wastewater, surface water, and groundwater worldwide, becoming then an anthropogenic marker of domestic waste and a contaminant of emerging concern (CEC). Regardless, the concentrations in which it is found are not toxic and it is easily removed by several technologies, as investigated by Bothfeld (2021). It has been proved that during the degradation of caffeine by oxidative purine catabolism, some plant metabolites such as xanthine, uric acid, allantoin, $NH_3$, and $CO_2$ are produced, contributing to nitrogen assimilation while modulating plant stress (Watanabe et al., 2010, 2014; Nourimand and Todd, 2016; Emanuil, 2022). In our study, caffeine treatment had a positive effect at the highest concentration and in irrigated plants only. Results showed a dose-effect response, in which the best results were observed at the highest dose tested. Furthermore, a detailed analysis of membrane lipid peroxidation showed enhanced MDA production in irrigated plants at sampling 2 (pod formation stage). Although this increase might be associated with an overall increased oxidative stress due to the most demanding reproductive stage, it is also very likely it may be associated with the fluctuating temperatures occurring in the greenhouse, with minimum daily temperatures attaining 2–3 °C during this period. Although the experiment was aimed at being performed under controlled conditions, the greenhouse used allowed regulation of photoperiod, but not of temperature at all, which decreased during the coolest nights of winter. Interestingly, this shows that irrigated plants were subjected to suboptimal conditions since low temperatures occurred during the most sensitive pheno-phase. This is in agreement with previous studies using other biostimulants (reviewed by Du Jardin, 2015 and Del Buono, 2021), some of them also related to nitrogen metabolism (Alfosea-Simón et al., 2020; Mesa et al., 2022), showing positive effects when plants are subject to suboptimal conditions or mild stress, but not severe stress (either biotic or abiotic). Since the European Biostimulant Industry Council (EBIC) defines biostimulants as substances that when applied stimulate natural processes (EBIC, 2019), caffeine might be used as a component of a biostimulant product in plants under natural fluctuating conditions. However, when stresses are too severe, our results suggest that we cannot indeed expect any biologically significant improvement caused by biostimulants, or in our case caffeine, because the plant is too severely damaged to positively respond to any external agent. Indeed, the same occurs with the human response to caffeine, in which this widely consumed psychoactive drug improves some physiological responses promoting arousal, alertness, energy, and elevated mood under suboptimal conditions or when stressful conditions are mild or very mild, but it exerts no effect or even negative effects when severe damage to organs occurs (Temple et al., 2017). It should also be noted that although lentil biomass produced per plant was only marginally significantly enhanced by caffeine treatment, these effects may represent important increases in yield that, if confirmed under field conditions, will lead to important economical revenues. As it has also been shown in other studies testing biostimulants in the similar pilot, greenhouse trials (Mesa et al., 2022), it is therefore of value to include results with a trend to statistically significant results, so that these positive effects can be scalable and therefore adequately tested in the field in future studies.

Fruit and seed development, a crucial growth phase in all grain crops, needs *de novo* synthesis of several components, long-distance photo-assimilate and nutrients transport, and biochemical processes that demand a large amount of energy utilization. Therefore, this development stage is one of the most sensible, especially to suboptimal growth conditions and environmental stresses, causing important crop yield reductions (Triboï et al., 2003; Barnabás et al., 2008; Ma et al., 2020). Drought is one of the major environmental stresses that adversely affect growth, physiology, and plant reproduction (Yordanov et al., 2000; Barnabás et al., 2008). Although lentil is considered to be relatively well adapted to drought, its high sensitivity to water deficit at different pheno-phases, such as flowering and pod formation stages, has been described (Mishra et al., 2014), causing important economic yield losses. Therefore, drought is one of the worst abiotic stresses for lentil production. Our findings align with these negative effects of drought on pod formation and seed filling, causing yield losses of around 65% in lentil plants. Moreover, our results also indicated that lentil production under water deficit was reduced by an increase in fruit abortion, which is one of the more limiting factors for achieving crop yield potential (McLaughlin and Boyer, 2004; Ruan et al., 2010). Biotic stress aborted pod filling in the same way as water deficit. However, lentil production loss was even more severe in plants parasitized by field dodder than by water deficit, reaching 80% losses compared to irrigated plants. Biotic stress generated by holoparasitic plants influences the growth and production of their hosts by extracting water, nutrients, and organic compounds from the host's vascular system, leading not only to drought stress in the host but also to nutrient starvation (Jiang et al., 2003; Shen et al., 2007; Prider et al., 2009). Our study showed that the sum of these underlying processes associated with parasitism was influencing the reproductive phenology of lentil plants in the last stages, accelerating fruit maturity and plant death. Stress-induced reductions in seed yield can be caused by different phenological alterations (Cleland et al., 2007), as we could observe in our study where a reduction in the length of this reproductive stage in parasited plants resulted in a decrease in filled pods.

Reproductive development was shown to be more sensitive to both stresses than vegetative growth in lentil plants but, eventually, reproductive and vegetative development are dependent on each other because efficient photosynthesis and reserve accumulation by the vegetative parts of the plant has a decisive role in the formation of new

reproductive organs (Blum et al., 1994). Our results showed a limited leaf expansion under drought conditions, which depends upon the turgor pressure and the supply of assimilates (Rucker et al., 1995). In turn, holoparasitism reduced the efficiency of photosystem II ($F_v/F_m$ ratio), which is in agreement with previous studies showing that host photosynthesis is suppressed by field dodder by limiting gas diffusion via stomatal and photosynthetic metabolic processes (Shen et al., 2007; Vrbničanin et al., 2013). These reductions were accompanied by enhanced MDA levels, thus suggesting oxidative damage. It is noteworthy that a transient increase in MDA already occurred at the first stages of the holoparasitic plant-host interaction, thus it is likely that this MDA plays a role in defense signaling (Weber et al., 2004). Conversely, the second MDA peak could be related to a sustained accumulation of MDA which trigger photosynthetic damage and cell death (Yamauchi et al., 2008). Similar oxidative behavior was observed in drought-stressed plants. Further research is however needed to disentangle the common and differential effects of MDA in plant response to low temperature alone (as it occurred in irrigated plants), or when this is superimposed to drought stress or holoparasitism (as it occurred in plants exposed to water deficit and field dodder, respectively).

## 5. Conclusions

Both water deficit and parasitism, and most particularly the latter, had a very negative impact on lentil production, effects that were not counterbalanced by caffeine application. Caffeine treatment positively influenced pod and lentil production at the highest dose and in irrigated plants only. Since these plants were exposed to remarkable growth temperatures fluctuations with low temperatures at night during reproductive development, it appears that caffeine application can exert a positive role when no more stresses are added (irrigated plants), but not in plants exposed to severe stress (either biotic or abiotic), thus indicating that environmental conditions and stress severity have a strong impact on the potential response of plants to caffeine when used as a potential ingredient for biostimulant formulations. Furthermore, foliar stress markers showed a much stronger sensitivity of lentil plants to biotic than abiotic stress, and a higher impact of both stresses on flowering and fruit formation than on the vegetative physiological status. Indeed, the positive effects of caffeine seemed to be more related to the number of fruits produced per plant (consequently leading to higher lentil production) than to any positive effect on the physiology of leaves, which suggests flower production, changes in plant architecture associated with flowering, or fruit set as possible underlying mechanisms explaining the observed positive effects of caffeine on lentil production, an aspect that warrants further investigations.

## CRediT authorship contribution statement

LJ, CM and SMB conceived the idea and designed experiments. LJ, CM and SC performed experiments. LJ performed statistical analyses and prepared the first draft of the manuscript. SMB revised the manuscript. All authors read and approved the final manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2022.105064.

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