Environmental and Experimental Botany 225 (2024) 105850

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Research paper

High ratio of blue:red light reduces fruit set in sweet pepper, which is associated with low starch content and hormonal changes

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ARTICLE INFO

Keywords:
Light spectrum
Fruit abortion
Carbohydrates
Phytohormones
Capsicum annuum L

ABSTRACT

In sweet pepper (*Capsicum annuum* L.), the fruit yield is often negatively affected by fruit abortion. Here we investigated whether fruit abortion is affected by the blue:red light ratio (B:R) and the possible underlying physiological mechanisms related to carbohydrates and hormones. Sweet pepper plants were grown at B:R of 1:10, 1:3, 1:1 or 9:1 with a total photosynthetic photon flux density of 200 μmol m^{-2} s^{-1}, resulting in a phytochrome photostationary state (PSS) of 0.88, 0.88, 0.86 and 0.72, respectively. For fruit set observations, each plant was allowed to retain 12 flowers on 4 main stems. Sweet pepper plants grown at the highest B:R (9:1) showed a low fruit set (around 3 fruits per plant), whereas the other three treatments resulted in higher fruit set (6–7 fruits per plant). This response matched with the changes in PSS, suggesting the B:R effect on fruit set might be controlled by phytochrome signaling, which requires further investigation. Plant shoot biomass and leaf area were reduced at B:R of 1:1 and 9:1. The reduced fruit set was associated with a drop in starch content and sucrose synthases activity; and a low auxin, high salicylic acid and high *cis*-Zeatin type levels in flowers. Flowers in the low fruit set treatment also failed to reduce the abscisic acid and ethylene levels after anthesis. We concluded that both the reduced starch content and the hormonal changes in flowers play a role in triggering fruit abortion at the high B:R of 9:1.

1. Introduction

Fruit set is a transition process where a flower develops into a fruit. It is a determining factor for the number of fruits on the plant, thus a major determinant of crop yield. Low fruit set, due to high flower and fruit abortion, limits yield in many crops including sweet pepper (*Capsicum annuum* L.). As an important vegetable crop, sweet pepper is cultivated worldwide (Russo, 2012). Even in protected cultivation, 70 %-80 % of young reproductive organs can abort in sweet pepper (Wubs et al., 2009). This results in not only a low yield but also a strong cyclical pattern in fruit set of sweet pepper, leading to unstable market supply and produce price (Heuvelink et al., 2004). Light spectrum can significantly affect fruit set and abortion. Decreasing the ratio of red to far-red light (R:FR) has been shown to induce more flower and fruit abortion in sweet pepper (Chen et al., 2022; Chen et al., 2024). Low R:FR is associated with a low photostationary state of phytochrome (PSS), a parameter that indicates the ratio of active phytochrome to total phytochrome. Along with blue and UV light receptors, phytochromes control plant morphogenesis, such as the shade-avoidance response (Legris et al., 2019). A low PSS value can be achieved by decreasing the R:FR ratio, but it also occurs under a spectrum with a very high blue:red light ratio (B:R). Therefore, it could be speculated that a very high B:R with a low PSS value induces flower and fruit abortion in sweet pepper, similar to a low R:FR ratio. Relating to this, shade avoidance responses at high fractions of blue light have been associated with low PSS (Kong et al., 2018; Kong et al., 2019).

As an abscission process, flower and fruit abortion in sweet pepper is tightly regulated by hormonal balances (Crane, 1969; Sawicki et al., 2015; Taylor and Whitelaw, 2001), where ethylene and abscisic acid are potential abscission accelerators, while auxin and gibberellin are

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https://doi.org/10.1016/j.envexpbot.2024.105850
Received 8 March 2024; Received in revised form 6 June 2024; Accepted 8 June 2024
Available online 13 June 2024

potential abscission inhibitors. B:R can potentially influence fruit set by regulating hormonal balances, such as through blue light receptors cryptochrome and phototropin. Activated cryptochromes led to a decreased abscisic acid concentration in wild-type *Arabidopsis* leaves compared to the *cry1cry2* mutant leaves (Boccalandro et al., 2012). Blue light postponed petal senescence of cutting carnation flowers, which was linked to the down-regulated expression of ethylene biosynthetic genes (Aalifar et al., 2020). This suggests that a high B:R may affect fruit set positively.

Apart from hormones, carbohydrates also play an important role in fruit set. In pepper flowers, decreased sugar accumulation was closely linked to more flower abortion (Aloni et al., 1996), possibly through enhancing abscisic acid and ethylene synthesis (Wien et al., 1989; Sawicki et al., 2015). Additional sugar in the cultivation media reduced abortion of pepper flower explants, probably by enhancing activity of sucrose synthases and invertases (Aloni et al., 1997), which are crucial enzymes in regulating fruit set and development (Nielsen et al., 1991; Ruan et al., 2012). Light spectrum can influence the carbohydrate status greatly (Heo et al., 2006). Cultivation under higher fractions of blue light leads to a lower starch content in leaves (Larsen et al., 2022; Shengxin et al., 2016); and a lower plant dry matter production (He et al., 2017; Wang et al., 2016; Warrington and Mitchell, 1976). This suggests that a high B:R may affect fruit set negatively by limiting photoassimilate availability.

Fruit set and the underlying mechanisms are often studied experimentally in controlled environment facilities, using genetic analyses, carbohydrates and hormones profiling, such as the studies conducted by Tiwari et al. (2012), (2013) and the studies reviewed by Ezura et al., (2023).

Considering the potential cross talk between carbohydrates and hormones, it remains inconclusive how B:R could influence fruit set. Spectra with blue and red light are commonly used as artificial lighting in indoor farming. However, to the best of our knowledge, the effect of different B:R on fruit set has not been studied so far. Here, we aimed to investigate the effect of a wide range of B:R on the fruit set of sweet pepper and the possible mechanisms. We hypothesized that increasing the B:R decreases fruit set, especially at very high B:R, where the PSS is lowered. We hypothesized this will be the result of lower plant dry matter production and lower carbohydrate accumulation in flowers, even with down-regulated ethylene and abscisic acid levels at high B:R. To investigate this hypothesis, sweet pepper plants were grown under four B:R of 1:10, 1:3, 1:1 or 9:1 in climate chambers, and various morphological and physiological parameters were observed.

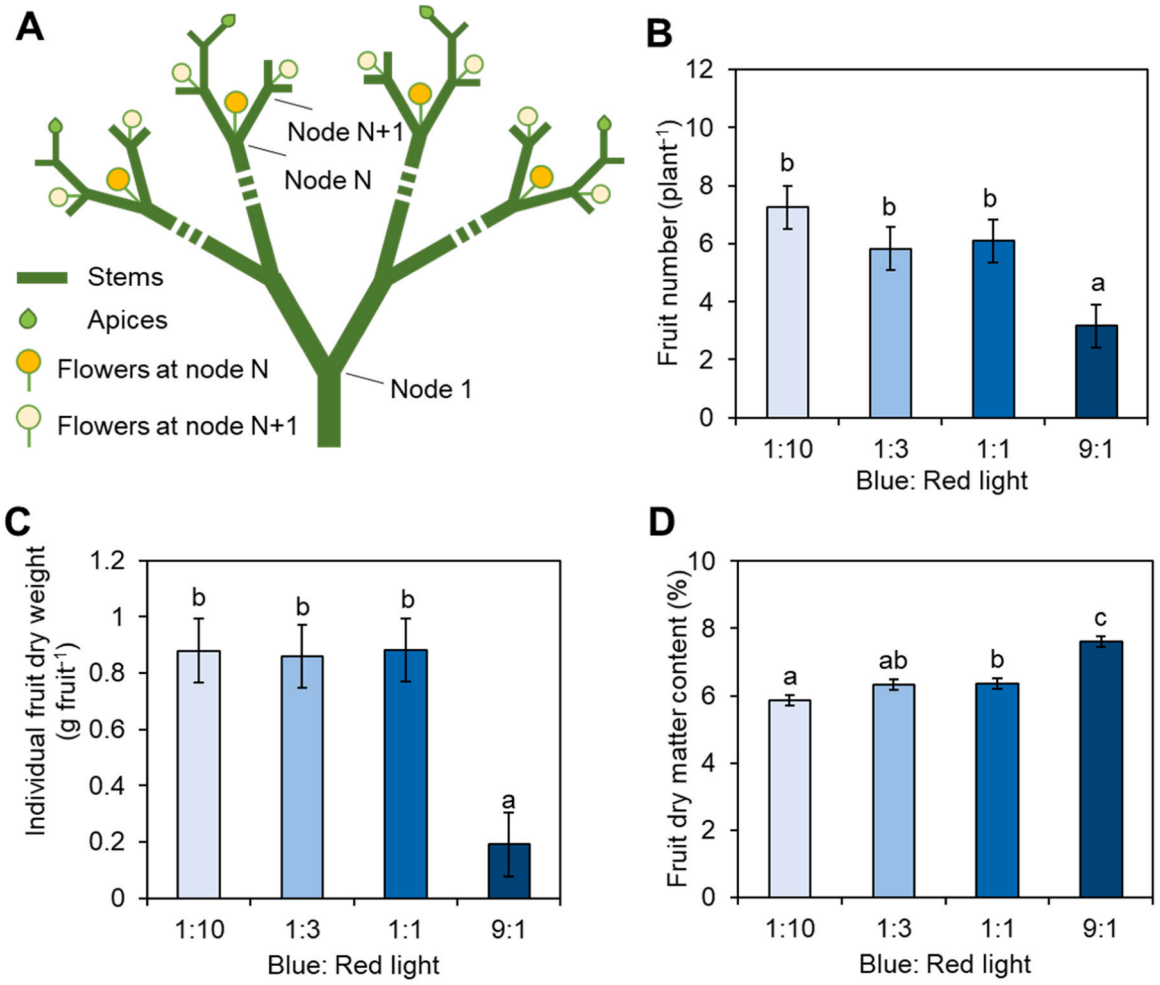

Fig. 1. Effect of blue:red light ratios (B:R) on fruit abortion and fruit growth in sweet pepper. (A) Plants were pruned to have 4 main shoots carrying 12 flowers per plant for fruit set observation. Node N=6 in the first replicate experiment, N=8 in the second replicate experiment. (B) Fruit number per plant. (C) Individual fruit dry weight. (D) Fruit dry matter content. Mean values were derived from 4 statistical replicates, each based on 5–6 plants. One-way ANOVA was performed on all variables. Error bars indicate ±standard error of means based on the common variance. Different letters indicate significant differences between treatment means according to Fisher's protected LSD test at P=0.05.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of sweet pepper (*Capsicum annuum* L. cv. Gialte, Enza Zaden, Enkhuizen, the Netherlands) were sown in potting mix soil (Lensli, Bleiswijk, the Netherlands), and transplanted into stonewool cubes (10×10×6.5 cm; Grodan, Roermond, the Netherlands) a week after germination. Seedlings were cultivated in a glasshouse (51°N, 5°E) for 7–8 weeks before moving into a climate chamber.

The experiment was conducted twice in time (seeds sown in July and September). The climate chamber was divided into eight cells: light treatments were randomly assigned to cells at the start of each replicate experiment. In each cell, 6 sweet pepper plants were grown on stonewool slabs with a density of 5.4 plants m^{-2}. The plants were pruned to maintain four main shoots (Fig. 1A). In the first replicate experiment, four flowers per plant at node 6 and eight flowers at node 7 were used for fruit set observations (Section 2.3), where nodes were counted from bottom to top with the first splitting node as node 1. In the second replicate experiment, flowers were two nodes higher, i.e., four flowers per plant at node 8 and eight flowers at node 9 were used for fruit set observations (Section 2.3), while four flowers at node 7 were sampled for lab analyses (Section 2.4). All other flowers were removed before their anthesis, and extra shoots were removed weekly when they were longer than 3 cm. Based on preliminary experiments, normal self-pollination was sufficient for good pollination thus no measures were taken to stimulate pollination.

Throughout the experiments, the average temperature was 21.9/18.3 °C (day/night) and the average humidity was 65 %. No CO_2 enrichment was applied. Nutrient solution (pH 6.0, EC 2.0 dS m^{-1}) was supplied through drip irrigation four times a day, which consisted of NO_3^- 12.4 mM, SO_4^{2-} 3.3 mM, HPO_4^{2-} 1.1 mM, NH_4^+ 1.2 mM, K^+ 7.2 mM, Ca^{2+} 4.1 mM, Mg^{2+} 1.8 mM, Fe^{3+}/Fe^{2+} 25 μM, Mn^{2+} 10 μM, Zn^{2+} 5 μM, $H_2BO_3^-$ 30 μM, Cu^+/Cu^{2+} 0.75 μM, MoO_4^{2-} 0.5 μM.

2.2. Light treatments

Plants in each cell were illuminated for 14 hours a day using dynamic LED panels (Phytofy® RL, OSRAM GmbH, Berlin, Germany). For the first week in the chamber, plants were grown under white light for acclimatization (Supplementary Table S1). After this, the light spectra were adjusted to the four treatment conditions consisting of only blue and red light with blue: red ratios of 1:10, 1:3, 1:1 or 9:1 (Table 1; Supplementary Fig. S1). The photosynthetic photon flux density (PPFD) of all treatments was about 200 μmol m^{-2} s^{-1} (400–700 nm) (Table 1). Light spectra were measured 20 cm below the LED panels with a spectrometer (LI-180, LI-COR Biosciences, Nebraska, USA). Light intensity was maintained by adjusting the height of the LED panels weekly to keep a constant 20 cm distance between the lamps and the top of the plant canopies.

In the first replicate experiment, the average anthesis date for flowers at nodes 6 and 7 were, respectively, 10 and 16 days after the start of light treatments. In the second replicate experiment, the average anthesis date for flowers at nodes 7 to 9 were, respectively, 8, 14 and 21 days after the start of the light treatments (Supplementary Table S2). Experiments ended about 20 days after the last anthesis: the first replicate experiment lasted 35 days, and the second replicate experiment lasted 42 days from the start of the light treatments.

Table 1
Four light treatments with different blue:red ratio (B:R). PPFD (photosynthetic photon flux density, 400-700 nm) were similar among treatments, where 400-500 nm was considered as blue light, and 600-700 nm as red light. B% and R% indicate the percentage of blue or red light in PPFD. In these light spectra, UV (380-400 nm) was 0.1-0.2 μmol m^{-2} s^{-1}; green (500-600 nm) was 0.4-0.6 μmol m^{-2} s^{-1}; and far-red (700-780 nm) was 0.03-1.2 μmol m^{-2} s^{-1}. The photoperiod was 14 hours. The mean ± standard error of the mean was derived from 4 replicates with 15 measurements per replicate. PSS (photostationary state of phytochrome) was determined according to Sager et al. (1988).

Blue: red ratio	Blue (μmol m^{-2} s^{-1})	Red (μmol m^{-2} s^{-1})	B%	R%	PSS	PPFD (μmol m^{-2} s^{-1})	DLI (mol m^{-2} d^{-1})
1:10	18 ± 2	182 ± 13	9.0	90.7	0.88	200 ± 15	10.1
1:3	50 ± 6	152 ± 11	24.8	75.0	0.88	203 ± 17	10.2
1:1	94 ± 11	101 ± 7	47.9	51.9	0.86	195 ± 18	9.8
9:1	175 ± 21	19 ± 1	90.0	9.8	0.72	195 ± 22	9.8

2.3. Morphological measurements

Anthesis and reproductive organ abortion were recorded every other day, where anthesis was defined as flower opening; flower abortion as the reproductive organ abscission before anthesis and fruit abortion as the reproductive organ abscission after anthesis. Fruit set was determined as the number of fruits obtained from 12 retained flowers per plant.

All plants were used for destructive morphological measurements at the end of a replicate experiment. Plant height was measured as the average height from stonewool surface to the apices of the four main stems per plant. Fruits, leaves and stems were separated. All leaves (including petioles) longer than 3 cm were used to determine the number of leaves, and the leaf area per plant was measured with a leaf area meter (Li-Cor LI-3100 C area meter). The fresh weight of each fruit (including the pedicel) was weighed immediately after removal. All plant materials were dried in a ventilated oven at 105°C for dry weight measurements: stems and leaves for 24 hours, and individual fruits for 72 hours.

2.4. Tissue sampling

In the second replicate experiment, four flowers per plant at node 7 were sampled for biochemical analyses (Sections 2.5, 2.6, 2.7), where each flower was sampled as a whole flower without separating reproductive organs. For each plant, two of the four flowers were collected at anthesis, while the other two were collected 7 days after anthesis. For each cell and developmental stage, flowers from 3 plants were pooled as one sample, resulting in two samples per stage per cell. Samples were collected in the middle of the light period (in the 7th hour of a 14-hour photoperiod). Samples were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

After two replicate experiments, a new batch of pepper plants was grown under the same set-up with a blue:red ratio of 1:10 or 9:1 to collect flowers at node 7–9 and top mature leaf samples for ethylene emission assay (Section 2.8) and pollen assessment (Section 2.9).

2.5. Carbohydrate quantification

The soluble sugars and starch were extracted from around 15 mg of freeze-dried tissue powder, using the same equipment as described by Min et al. (2021), with some adaptations as described by Chen et al. (2024). In short, carbohydrates were extracted with 5 ml 80 % ethanol (v/v) in an 80 °C water bath for 20 min. After centrifuging at 4 °C, the supernatant was vacuum dried (Savant SpeedVac SPD2010, Thermo Fisher Inc.), then dissolved in Milli-Q water to quantify soluble sugars. The pellet was vacuum dried, then incubated together with 2 ml alpha-amylase solution (1 mg ml^{-1}) at 90 °C for 30 min. Then, 1 ml amyloglucosidase (0.5 mg ml^{-1} in 50 mM citrate buffer, pH 4.6) was added, followed by an incubation at 60 °C for 10 min. The samples were then centrifuged at 4 °C, where the supernatant was used to quantify starch as glucose. The carbohydrates were quantified with a high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Dionex™ ICS-5000, Thermo

Fisher Scientific), equipped with a Dionex™ CarboPac™ PA1 column (2 ×250 mm; Thermo Fisher Scientific).

2.6. Enzyme assay

The methods to determine the activity of invertases and sucrose synthases were adapted from Aloni et al. (1991), (1996), as described by Chen et al. (2024). In short, around 100 mg (fresh weight) finely ground samples were homogenized in 1.5 ml ice-cold extraction buffer (pH 7.2) containing: 25 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid), 5 mM $MgCl_2$, 0.5 mM Na_2EDTA (Ethylenediaminetetra-acetic acid disodium salt), 2 mM DDT (DL-Dithiothreitol), 3 mM DIDCA (Diethyldithiocarbamic acid), 1 % (w/v) PVP (Polyvinylpyrrolidone), and 0.1 % (v/v) Triton X-100. The mixture was centrifuged at 21300 rcf for 20 min at 4 °C, where the supernatant was used to determine protein content with the Bradford test. The activity of soluble acid invertase or neutral invertase was determined with a 50 µl aliquot of the supernatant incubated in 500 µl 0.1 N phosphate citrate buffer (pH 5.0 or pH 7.5 respectively) with 20 mM sucrose. The activity of insoluble cell wall invertase was determined with a 50 µl aliquot of the suspended pellet incubated in 500 µl 0.1 N phosphate citrate buffer (pH 5.0) with 20 mM sucrose. The activity of sucrose synthase was determined as sucrose breakdown during aliquots of 50 µl of the supernatant incubated in 500 µl 0.1 N phosphate citrate buffer (pH 7.0) with 200 mM sucrose and 5 mM UDP (uridine 5′-diphosphate). Boiled enzymes were used as the blank for each reaction. After incubation at 37 °C for 60 min, the resulting reducing sugars was determined colorimetrically at 540 nm by the dinitrosalicylic acid reaction. The activity of enzymes was expressed as the amount of reaction products per hour on fresh weight basis.

2.7. Hormonal profiling

Ground frozen samples (around 20 mg fresh weight) were extracted with 1 ml 100 % methanol containing the appropriate internal standards (Supplementary Table S3) as previously described (Schiessl et al., 2019; Gühl et al., 2021) with minor modifications. Column flow through and wash (formic acid fraction) was collected and used to quantify ACC (1-aminocyclopropane-1-carboxylate) concentration as previously described by Bours et al., (2013). The concentration of all plant hormones was determined by Multiple Reaction Monitoring Ultra Performance Liquid Chromatography and Tandem Mass Spectrometry (MRM-UPLC-MS/MS) (Waters, Milford, USA) as previously described. Parent–daughter transitions for the different compounds were set using the IntelliStart MS Console. MRM transitions selected for compound identification and quantification are shown in Supplementary Table S3, a 10-point calibration curve was constructed for each compound ranging from 0.1 µM to 19 pM and each dilution also contained a known amount of an appropriate deuterium-labelled internal standard.

2.8. Ethylene emission assay

Flowers were collected at two developmental stages: 1–3 days after anthesis or 4–7 days after anthesis (six replicates per stage). Leaf discs were sampled with a cork borer of 1.5 cm diameter (two replicates). The samples were collected in the morning and incubated in sealed 20 ml glass vials at room temperature (about 20°C). Each statistical replicate consisted of 3 glass vials, where each had 4–5 individual flowers or 5 leaf discs. After 5 hours of incubation, 3 ml air samples from the vials were analysed with a Trace-1300 Gas Chromatogram (InterScience, Breda, NL) coupled with a flame ionization detector, as described by Torres Ascurra et al. (2023). Gas Chromatogram was calibrated with a set of certified calibration gases (SOL group, Monza, Italy) before measurements.

2.9. Pollen quantity and viability assessment

Flowers were collected at anthesis for pollen assessment. Anthers from each flower were first fixed in Carnoy's fixative. After the anthers were dried and stained with Alexander's dye, the pollen were released by ultrasonic bath for 5 min, and further observed with a Fuchs-Rosenthal hemocytometer (4 ×4×0.2 mm grid, 3.2 mm^3) under a stereoscope (LEICA MZ APO). Pollen with a magenta red color were scored as viable, while pollen with a blue-green color were scored as aborted. Observation of each flower included 2 technical replicates with 6 views each. The total number of pollen per grid in each view was recorded to calculate the number of pollen per flower, based on the ratio of solution volume between the space within each grid and the total dyeing solution used per flower. There were four replicates, where each replicate consisted of 8 individual flowers.

2.10. Statistical analysis

Each light treatment had 4 statistical replicates, composed of 2 replicate cells in each of two replicate experiments. Data that had been assessed on several plants per replicate cell were first averaged to give one value per cell (experimental unit). The first 2 statistical replicates each consisted of 5 plants, and the second 2 statistical replicates each consisted of 6 plants. Statistics were performed with Genstat software (21st edition). One-way Analysis of Variance (ANOVA) with randomized blocks, where four replicates were considered as four blocks, was used to determine significant differences in morphological parameters among the four light treatments. Fisher's protected least significant difference (LSD) test at P=0.05 was used for mean separation. For enzymatic activity, carbohydrate and hormone quantification, ANOVA with split-plot design was used (light treatments as whole plots, developmental stages as sub-plots), where Fisher's unprotected LSD test at P=0.05 was used, due to the insignificant interactions between both factors for some parameters. Homogeneity of variances was assumed, and normality of residuals was tested with Shapiro-Wilk test at P=0.05 level. If the residuals in ANOVA analysis did not follow normal distribution, the log-transformed data were used for statistical tests. This applied to the concentration of ACC (1-amino-cyclopropane-1-carboxylic acid) and SA (salicylic acid), where their residuals in ANOVA with log-transformed data showed normal distribution.

3. Results

3.1. Fruit set was reduced by the highest blue:red light ratio

Sweet pepper plants, cultivated under four different blue:red light ratios (B:R), were pruned to have only 12 flowers per plant to observe flower and fruit abortion (Fig. 1A; plant photos - Supplementary Fig. S2). The number of days to anthesis was unaffected by B:R (Supplementary Table S2), with almost no flower abortion before anthesis. Hence, the B:R effect on fruit abortion and growth was mainly post-anthesis.

Fruit number and individual fruit dry weight were similar at B:R of 1:10, 1:3 or 1:1. However, at B:R of 9:1, the fruit number was almost reduced by half and the individual fruit dry weight was about 75 % lower compared to the lower B:R (Fig. 1B, C). In contrast, the fruit dry matter content gradually increased with increasing B:R and was highest at B:R of 9:1 (Fig. 1D). Fruit abortion at B:R of 1:10 was delayed by 1–1.5 days compared to the higher B:R (from 1:3 to 9:1) (Supplementary Table S4). Fruit abortion was not affected by the number of pollen per flower or the fraction of viable pollen, as these were not influenced by B:R (Supplementary Fig. S3). There was no visual difference in the seed number of harvested fruits.

3.2. Higher blue:red light ratios resulted in a lower plant biomass with a lower leaf area

Total shoot biomass was similar for plants at B:R of 1:10 and 1:3, but decreased when B:R increased further (Fig. 2A). The reduction of shoot biomass at B:R of 1:1 was the result of a lower leaf and stem biomass, while at B:R of 9:1, that was the result of lower leaf, stem, and fruit biomass. The dry mass partitioning among plant organs was not influenced by B:R from 1:10 to 1:1, but at 9:1, the partitioning to fruits substantially dropped (Fig. 2B).

The leaf area per plant was reduced at B:R of 1:1 and 9:1, compared to lower B:R (Fig. 2C). Considering that leaf number was not affected by B:R (Supplementary Fig. S4 A), we suggest that high B:R inhibited leaf expansion in pepper. The specific leaf area and plant height were unaffected by B:R (Supplementary Fig. S4 B, C).

Most fruit abortion was observed between 9 and 16 days after anthesis (Supplementary Table S4). Thus, the carbohydrate content was analyzed in the flowers on day 0 (at anthesis) and fruits on day 7 after anthesis, which could indicate the carbohydrate status before potential abortion. The carbohydrate content (fructose, glucose, sucrose, and starch) generally dropped on day 7 after anthesis compared to at anthesis (Fig. 3). At both developmental stages, sucrose, fructose, and glucose content were not influenced by B:R (Fig. 3A, B, C). However, starch content at both stages was reduced at B:R of 9:1 compared to the other B:R (Fig. 3D).

3.3. Effect of blue:red light ratios on enzyme activity for sucrose cleavage

The activity of soluble acid invertases, cell wall invertases and neutral invertases in flowers/fruits showed a substantial decline on day 7 after anthesis compared to at anthesis (Fig. 4A, B, C). At anthesis, the activities of these enzymes were not influenced by B:R. On day 7 after anthesis, the soluble acid invertase activity was lower than the other three B:R; the cell wall invertase activity was higher at B:R of 9:1 compared to the B:R of 1:3 and 1:1; whilst the neutral invertase activity was unaffected by B:R (Fig. 4A, B, C).

The activity of sucrose synthases showed different patterns among B:R at the two developmental stages. At anthesis, sucrose synthase activity increased along with the increasing B:R from 1:10 to 1:1, but this tendency did not continue - the sucrose synthase activity showed a substantial drop at B:R of 9:1 (Fig. 4D). On day 7 after anthesis, the sucrose synthase activity was higher at B:R of 9:1 compared to the B:R of 1:3 and 1:1 (Fig. 4D).

In addition, the protein content on day 7 after anthesis was substantially lower at B:R of 9:1 compared to the other B:R, whilst the protein content at anthesis was unaffected by B:R (Supplementary Fig. S5).

3.4. Effect of blue:red light ratios on the hormone concentrations

The effect of B:R on hormone concentrations in flowers/fruits differed between two developmental stages (Fig. 5; Supplementary Fig. S6). At anthesis, the concentration of IAA (indole-3-Acetic Acid) and

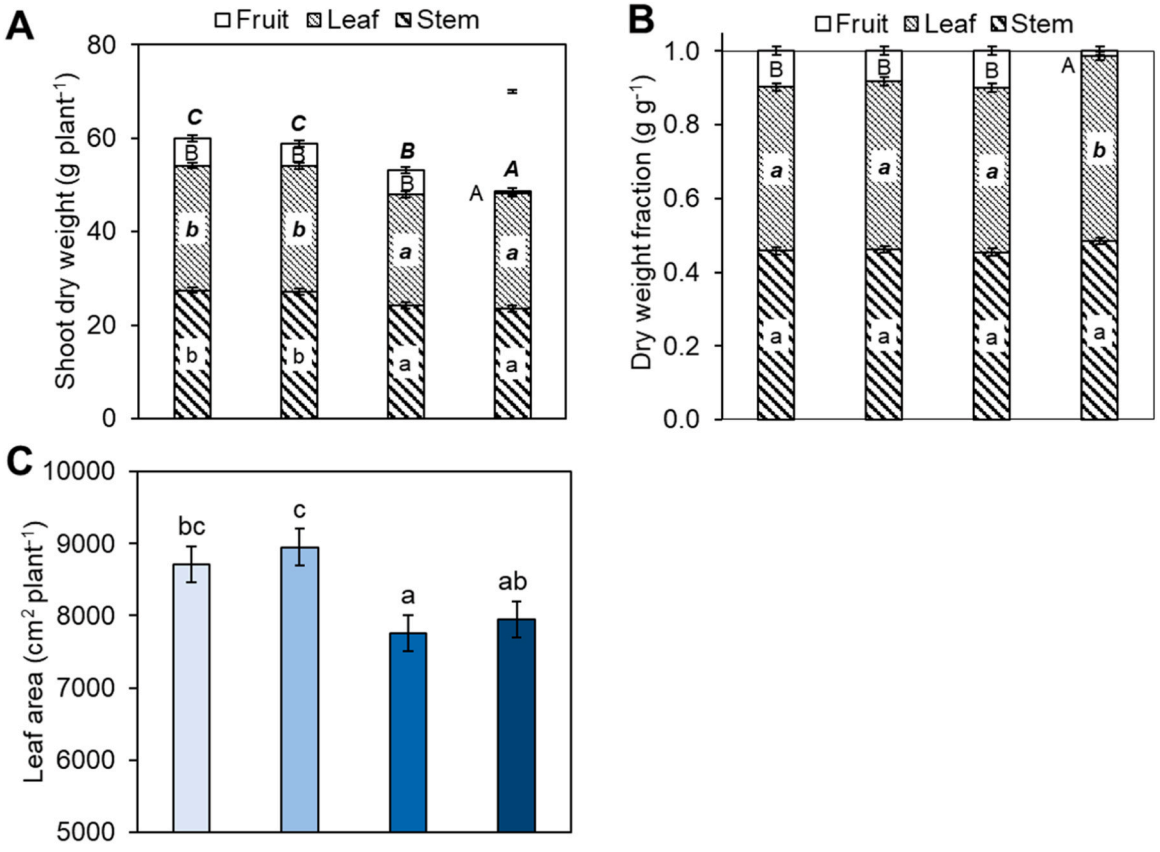

Fig. 2. Effect of blue:red light ratios (B:R) on plant morphology in sweet pepper. (A) Dry weight of stems, leaves and fruits per plant, where the sum was defined as total shoot dry weight. (B) The fraction of dry mass partitioned to stem, leaf and fruit. (C) Leaf area per plant. For all variables, mean values were derived from 4 statistical replicates, each based on 5–6 plants. One way ANOVA was performed for all variables. Error bars indicate ±standard error of means based on the common variance. Different letters indicate significant differences between treatment means according to Fisher's protected LSD test at P=0.05. In (A), the additional error bar at the top right corner indicates the standard error of means for total shoot dry weight. In (A) and (B), standard small letters are for stem, small letters in bold italics are for leaves, standard capital letters are for fruits and capital letters in bold italics are for total shoot. ***3.3 The highest blue:red light ratio reduces starch content in flowers***.

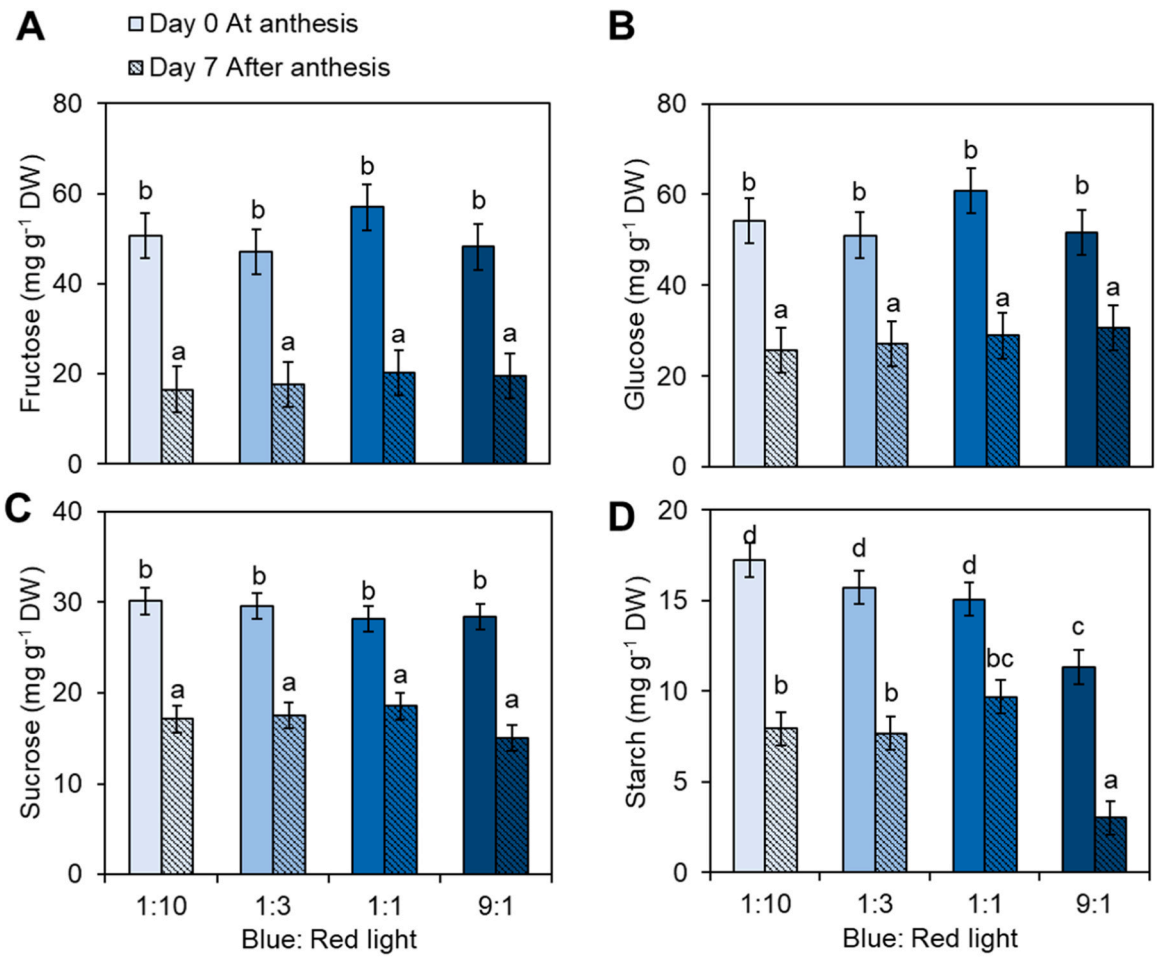

Fig. 3. Carbohydrate content of pepper flowers on day 0 (at anthesis) and fruits on day 7 after anthesis. Samples were collected in the middle of the day (in the 7th hour of a 14-hour photoperiod). DW stands for dry weight. Mean values were derived from 2 statistical replicates, each based on 2 samples (3 plants/sample). Split-plot ANOVA was performed with light treatments as the whole plot and developmental stages as the sub-plot. Error bars indicate ±standard error of means based on the common variance. Different letters indicate significant differences between treatment means according to Fisher's unprotected LSD test at P=0.05.

SA (salicylic acid) were not influenced by B:R (Fig. 5C,D). IAA concentration on day 7 after anthesis was about three times higher compared to at anthesis. SA concentration did not differ between these two stages. This pattern of IAA and SA applies to all B:R except at B:R of 9:1. At B:R of 9:1, IAA was reduced, and SA was doubled on day 7 after anthesis, compared to the other B:R (Fig. 5C,D).

ABA (abscisic acid) concentration was not influenced by B:R at anthesis. Compared to anthesis, the concentration of ABA on day 7 after anthesis decreased and this reduction was less pronounced at the higher B:R. This resulted in a higher ABA concentration on day 7 after anthesis at B:R of 9:1, compared to B:R of 1:10 and 1:3 (Fig. 5A).

At anthesis, B:R influenced neither the concentration of the precursor of ethylene - ACC (1-aminocyclopropane-1-carboxylate), nor the ethylene emission rate (Fig. 5B, E). On day 7 after anthesis, ACC showed a high concentration at B:R of 1:10, which decreased with increasing B:R, showing similarly low levels at B:R of 1:1 and 9:1 (Fig. 5B). After anthesis (from day 1–3 to 4–7 after anthesis), ethylene emission from flowers/fruits was reduced at B:R of 1:10, but not at B:R of 9:1, where ethylene emission maintained at a high level (Fig. 5E). The ethylene emission rates from leaves were not influenced by B:R (Fig. 5F).

The hormone concentrations in the jasmonic acid pathway, including jasmonic acid (JA), jasmonic acid isoleucine (JA-ile, the main active jasmonate), and 12-oxo-Phytodienoic Acid (OPDA, main jasmonate precursor), were not influenced by B:R but differed only between developmental stages (Supplementary Fig. S6), where JA and JA-ile decreased, and OPDA increased on day 7 after anthesis compared to at anthesis.

Different forms of cytokinins (iPR, iP, *c*ZR, *c*Z, *t*Z, *t*ZR) at anthesis were generally not influenced by B:R (Supplementary Fig. S6). On day 7 after anthesis, B:R of 9:1 led to a substantially higher concentration of *c*ZR and *c*Z compared to the other B:R, while B:R did not influence the concentration of the other forms of cytokinins (Supplementary Fig. S6).

4. Discussion

4.1. Low fruit set at the very high ratio of blue:red light might be regulated by phytochromes

In this study, we aimed to investigate the effects of different blue:red light ratios (B:R) on the fruit set in sweet pepper and explain effects from physiological and morphological perspectives.

We found the fruit set was unchanged among B:R from 1:10 to 1:1, but it decreased at the highest B:R of 9:1 (Fig. 1B). This pattern, with only the B:R of 9:1 showing a different response, also appeared in the reduced individual fruit weight (Fig. 1C); the reduced starch content in flowers (Fig. 3D); the reduced activity of soluble acid invertases after anthesis (Fig. 4A); the reduced auxin (IAA), the increased salicylic acid (SA) and the increased cytokinin *c*Z and *c*ZR concentrations after anthesis (Fig. 5C,D; Supplementary Fig. S6). These observations are consistent with the changes of PSS (photostationary state of phytochrome) among our treatments: increasing the B:R from 1:10 to 1:1 minimally changes the PSS (0.86–0.88) but increasing the B:R further to

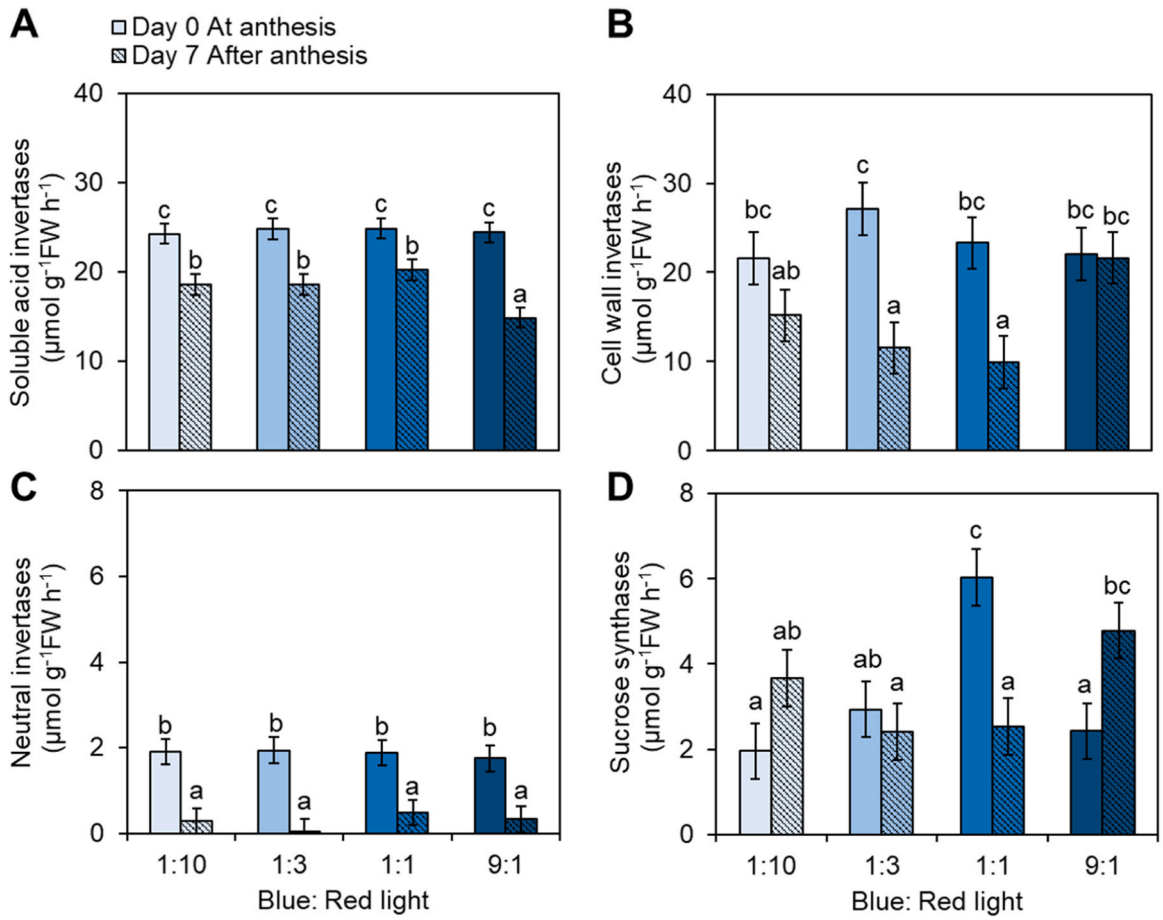

Fig. 4. Enzyme activity of invertases and sucrose synthases in pepper flowers on day 0 (at anthesis) and fruits on day 7 after anthesis. Samples were collected in the middle of the day (in the 7th hour of a 14-hour photoperiod). FW stands for fresh weight. Mean values were derived from 2 statistical replicates, each based on 2 samples (3 plants/sample). Split-plot ANOVA was performed with light treatments as the whole plot and developmental stages as the sub-plot. Error bars indicate ±standard error of means based on the common variance. Different letters indicate significant differences between treatment means according to Fisher's unprotected LSD test at P=0.05.

9:1 led to a substantial decrease of PSS to 0.72 (Table 1). Thus, we speculate the mentioned observations to be regulated by phytochrome controlled pathways.

In previous study, we observed that additional FR, hence reduced PSS, also caused a reduced fruit set in sweet pepper (Chen et al., 2022), which was also associated with a reduced activity of soluble acid invertases, a reduced IAA concentration, and an increased SA concentration in fruits 7 days after anthesis (Chen et al., 2024). These shared responses suggest that the low fruit set at B:R of 9:1 might be mediated by phytochromes too. To further disentangle the roles of phytochrome and blue light receptors in this effect, fruit set should be compared among light spectra with the same low PSS obtained by different fractions of blue light supplemented with FR in a future study. In addition, more B:R levels should be investigated (especially between B:R of 1:1 to 9:1) supplemented with transcriptional analysis on phytochrome and blue light receptor response genes, to better understand the relationship between fruit set, PSS, carbohydrates and hormonal dynamics.

4.2. *Higher B:R reduces plant source strength*

High B:R of 1:1 and 9:1 reduced shoot biomass of sweet pepper plants by around 11 % and 18 % respectively, compared to lower B:R (Fig. 2A). This biomass reduction could partially be attributed to a 9–13 % lower leaf area at B:R of 1:1 and 9:1 (Fig. 2C). Since leaf area and plant biomass decreased from B:R of 1:3 to 1:1, these responses may have been regulated by cryptochrome and phototropin photoreceptors, especially because the PSS minimally changed in this range (Table 1). It is widely reported that increasing the fraction of blue light decreases leaf area (Cope et al., 2014; Kalaitzoglou et al., 2021; Kusuma et al., 2021; Larsen et al., 2020; Snowden et al., 2016), although the presence or magnitude of the response can be species and environment dependent. The reduced leaf area could lead to a reduced light interception at B:R of 1:1 and 9:1.

In addition to the decrease in light interception, blue photons tend to have a slightly lower photosynthetic efficiency than red photons when measured on the same plant (McCree, 1971; Zhen and Bugbee, 2020; Hogewoning et al., 2012). However, it is important to emphasize potential acclimation to B:R, which affects the photosynthetic response. Increasing B:R also tends to increase leaf thickness, chlorophyll content, stomatal density and conductance, which can contrastingly result in increasing photosynthetic efficiencies on a leaf area basis (Wang et al., 2016; Hogewoning et al., 2010). Therefore, the effect of B:R on photosynthesis may be complex, where the photosynthesis may be optimized under some moderate B:R. For example, the highest net photosynthesis rate was shown at B:R of 1:4 in lettuce (Kang et al., 2016), at B:R of 3:2 in cherry tomato (Liu et al., 2018), and at B:R of 1:3 in sweet pepper (Li et al., 2023). This could possibly explain the increased leaf photosynthesis rate with the increasing B:R ratios reported by some researchers, especially below B:R of 1:1 (Wang et al., 2016; Hogewoning et al., 2010). Responses for higher B:R ratios (between 1:1 and 1:0) are rarely studied. Compared to a more balanced B:R, monochromatic blue light can significantly reduce both acclimated and non-acclimated photosynthesis (Hogewoning et al., 2010; Zhen and Bugbee, 2020). The

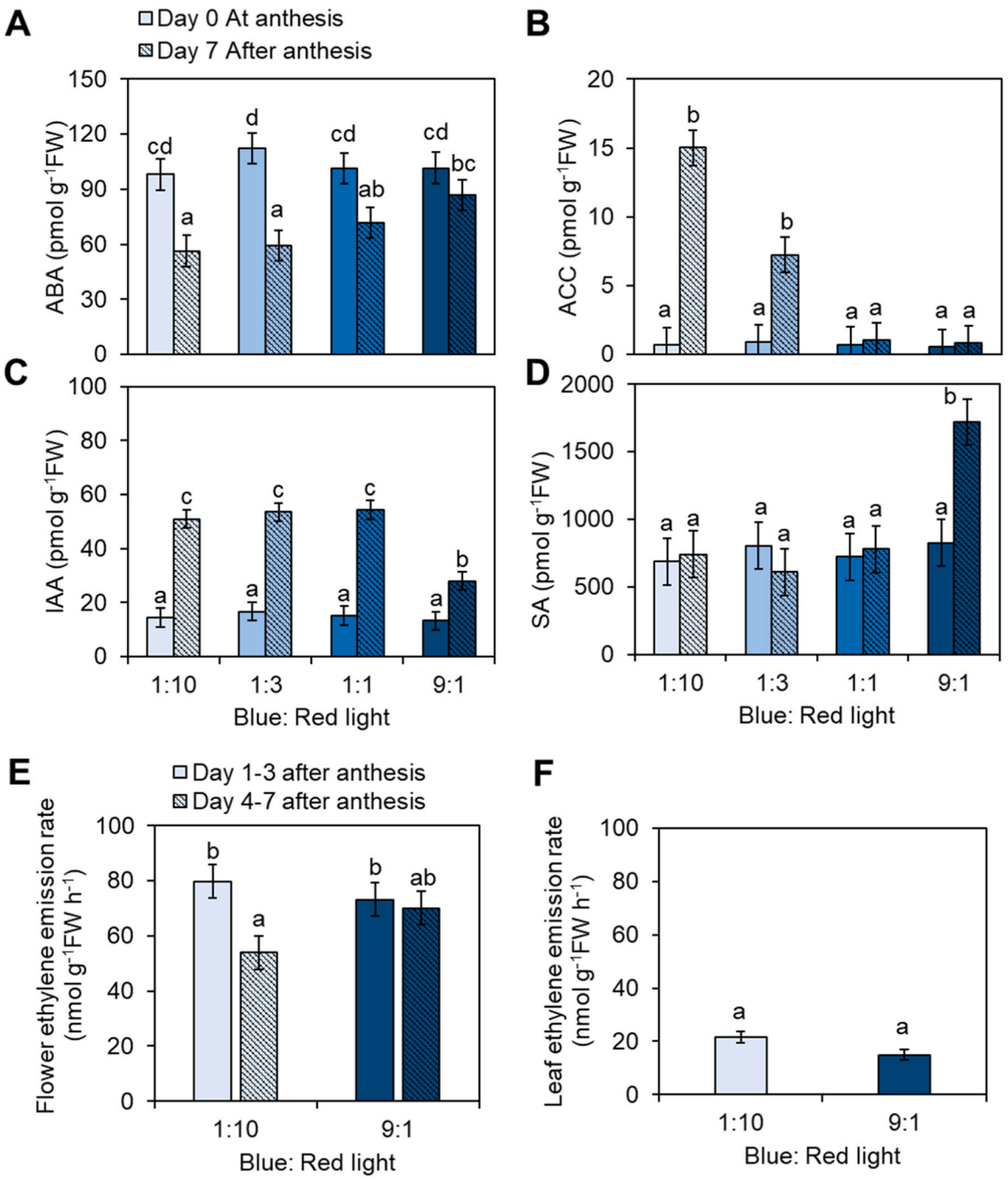

Fig. 5. Hormone concentrations in pepper flowers on day 0 (at anthesis) and fruits on day 7 after anthesis. FW stands for fresh weight. For (A) ABA (abscisic acid), (B) ACC (1-amino-cyclopropane-1-carboxylic acid), (C) IAA (indole-3-Acetic Acid) and (D) SA (salicylic acid), mean values were derived from 2 statistical replicates, each based on 2 samples (3 plants/sample). (A)-(D) Samples were collected in the middle of the day (in the 7th hour of a 14-hour photoperiod). For ethylene emission from flowers (E) and leaves (F), the mean values were derived from 6 replicates for flowers, and 2 replicates for leaves. (*E*)-(F) Each replicate was based on 3 glass vials with 4–5 flowers or 5 leaf discs per glass vial. Samples were collected in the morning. Split-plot ANOVA was performed for (A)–(E) with light treatments as the whole plot and developmental stages as the sub-plot; one way ANOVA was performed for (F). Error bars indicate ±standard error of means based on the common variance. Different letters indicate significant differences between treatment means according to Fisher's unprotected LSD test at P=0.05. Log-transformed data was used for mean separation in (B) and (D) as the residuals in ANOVA analysis based on their original data did not follow normal distribution.

highest B:R (9:1) in the current study is close to monochromatic blue, thus, we speculate that such high B:R could possibly reduce net photosynthesis rate in sweet pepper. Therefore, the reduced plant dry matter production could result from both the lower leaf area and potentially lower photosynthesis rate at the highest B:R.

4.3. Low fruit set is associated with low starch content

High B:R of 9:1 caused a lower starch content in flowers (Fig. 3D). In many perennial species, flowers with sufficiently available sugar at anthesis, especially high starch content, are more likely to develop into fruits successfully (in apricot: Rodrigo et al., 2000; in avocado: Alcaraz et al., 2013; Boldingh et al., 2016; in grapevine: Lebon et al., 2008). The stronger capability to accumulate sugars in flowers, especially starch, is

related to a lower sensitivity to flower and fruit abortion among cultivars of grapevine (Lebon et al., 2004) and sweet pepper (Aloni et al., 1996). Thus, the lower floral starch content could be one of the main triggers for the reduced fruit set at very high B:R (Fig. 1B).

The starch content in flowers and fruits could be limited by starch accumulation in source and storage organs. A higher starch content in fruiting branches and source leaves is correlated with a higher fruit set in citrus and avocado (Schaffer et al., 1985; Davie et al., 1995). A higher fraction of blue light (90 % blue vs. 9 % blue) was found to reduce starch content in basil leaves (Larsen et al., 2022). Blue light signal via cryptochrome 1a was found to induce starch degradation in tomato leaves and lead to less starch accumulation (Dong et al., 2021). Thus, high B:R may limit starch accumulation in source organs, and limit assimilate translocation from source to sink organs, e.g., flowers and fruits. The reduced plant dry matter production (discussed in Section 4.2), which indicates a reduced source strength, also implies a lower assimilate availability for flowers.

Other than assimilate availability, the starch synthesis and accumulation in the flowers is largely dependent on the SuSy (sucrose synthases) activity (Angeles-Núñez and Tiessen, 2010; Baroja-Fernández et al., 2009; Baroja-Fernández et al., 2012; D'Aoust et al., 1999; N'tchobo et al., 1999). In tomato fruit, a reduced SuSy activity was correlated with a reduced starch content, which led to a reduced sucrose unloading capacity in young fruit, a slower fruit growth rate and a reduced fruit set (D'Aoust et al., 1999). In flowers at anthesis, the activity of sucrose synthases gradually increased from a B:R of 1:10 to 1:1, followed by a substantial drop at B:R of 9:1 (Fig. 4D). This drop matches the reduced floral starch content at B:R of 9:1 (Fig. 3D). The increased SuSy activity from B:R of 1:10 to 1:1 could be a response of the flowers to the decreasing source strength, resulting in the need to secure assimilates. Our data suggest that a drop in sucrose synthase activity at anthesis, in combination with a reduction in plant source strength, was probably responsible for the reduced floral starch content at the highest B:R.

4.4. The role of hormones in the reduced fruit set at the highest B:R

High B:R of 9:1 led to changes in hormones in flowers and fruits before potential abortion, where the abortion was mostly observed between 9 and 16 days after anthesis. Auxin (IAA) together with gibberellins act as stimulating signals for fruit set and the subsequent activation of cell division (in tomato Vriezen et al., 2008; in pepper Tiwari et al., 2012), thus the lower IAA level at B:R of 9:1 was in line with the reduced fruit set compared to the other B:R. With antagonistic roles to auxin and gibberellin, ethylene and abscisic acid (ABA) are also involved in regulating fruit set. Their biosynthesis genes were strongly expressed before fruit set but attenuated soon after fruit set (Vriezen et al., 2008). We found that ABA concentration and ethylene emission in fruits on day 7 after anthesis were reduced at low B:R but remained at the high level at B:R of 9:1 (Fig. 5A, E). This supports the roles of ABA and ethylene in promoting abscission of reproductive organs (Lee et al., 2021; Shinozaki et al., 2015; Wilmowicz et al., 2016). Interestingly, the concentration of ethylene precursor ACC showed a contradictory pattern to the ethylene emission rate in flowers: from anthesis to 7 days after anthesis, the ethylene emission was reduced but ACC was increased at low B:R; whilst the ethylene emission stayed high, and ACC stayed low at high B:R (Fig. 5B, E). This implies the ethylene production via ACC oxidase (Wang et al., 2002) could be one of the processes regulated by B:R. At low B:R, the reduced ethylene production after anthesis could result in more ACC accumulation.

To the best of our knowledge, no research shows a direct role of salicylic acid (SA) in fruit set. Patharkar et al. (2017) and Patharkar and Walker (2018) suggested that SA could be involved in regulating abscission. High B:R could be stressful for plants. A short blue light illumination may increase the formation of reactive oxygen species (ROS) via cryptochrome activation (El-Esawi et al., 2017), which correlates to the increase of cell death in plant protoplasts (Consentino et al., 2015). High B:R in the current study might have resulted in ROS bursts, which could have triggered SA signaling and programmed cell death (Herrera-Vásquez et al., 2015; Lukan and Coll, 2022). However, El-Esawi et al. (2017) suggested that under steady state, long term growth conditions, the intracellular levels of ROS are unlikely changed by cryptochrome-induced ROS production, due to a tightly regulated redox state equilibrium. Thus it remains doubtful whether the high SA levels at B:R of 9:1 resulted from a possible oxidative stress. Moreover, a contrasting report showed that blue light reduces SA level by down-regulating its biosynthesis in broccoli sprouts compared to white or red light (Wang et al., 2022). Therefore, further investigations will be required to determine the role of SA in the effect of B:R on fruit set, and its relation to oxidative stress. Other than blue light receptors, SA may also be under the control of phytochromes (as discussed in Section 4.1), considering SA pathway genes are found as key components in shade avoidance responses (Nozue et al., 2018).

The roles of cytokinins (CKs) in fruit set are broad: CKs stimulate cell division in developing fruits (Vriezen et al., 2008), initiate parthenocarpic fruit set (Sharif et al., 2022), but stimulate lemon pistil abscission in vitro (Sipes and Einset, 1983), and trigger seed abortion when seeds perceive sugar depletion (Botton et al., 2011). The different roles of CKs could relate to the CK type and localization, tissue developmental stage and plant species. High B:R of 9:1 increased only *c*Z and *c*ZR, both belonging to the *cis*-Zeatin type of cytokinins (Supplementary Fig. S6). Higher levels of *c*Z and *c*ZR could suggest a higher biosynthesis through turnover or degradation of tRNAs (Schäfer et al., 2015), which is a different biosynthesis pathway than *trans*-Zeatin type (*t*Z, *t*ZR) and the isopentenyladenine type (iP, iPR) cytokinins. *c*Z type is thought to be much less biologically active compared to *t*Z or iP types. Even though the functions of *c*Z and *c*ZR are not fully clear, their accumulation has been associated with limited growth (Gajdošová et al., 2011; Schäfer et al., 2015). This is in line with the lower individual fruit biomass found at the highest B:R (Fig. 1C). Despite a lack of experimental evidence, Schäfer et al. (2015) hypothesized in their review that, *c*Z and *c*ZR would become abundant (instead of *t*Z and *t*ZR) under growth-inhibiting conditions (e.g., in response to stress or at certain developmental stages), to maintain a minimal CK activity necessary for plant survival and subsequent recovery. The highest B:R in our study might be perceived as a growth-inhibiting condition for pepper flowers.

It is noteworthy that hormones and carbohydrates were quantified from a pooled collection of flowers before potential abortion took place, and as such some of these flowers may have set fruit while others aborted (see Fig. 1B for approximate fruit set out of 12 flowers). Therefore, hormone and carbohydrate concentrations in aborted and non-aborted flowers may be more extreme than values presented here (Fig. 3, Fig. 5, Supplementary Fig. S6), however we believe that the general trends still stand.

Taken together, the low IAA, high SA, high *c*Z and *c*ZR, the unreduced ABA and ethylene at the highest B:R, seem to indicate the trade-off balance of flowers is leaning towards defense and dormancy instead of growth (Huot et al., 2014). We suggest this change could potentially trigger more fruit abortion at very high B:R. Considering the hormonal change was only found on day 7 after anthesis but not at anthesis, we suspect it to be a possible downstream response to the low floral starch content at anthesis. However, to improve the understanding of the dynamics of hormonal regulation in this response, and how it is related to carbohydrates metabolism at different reproductive organs, further investigations are required to increase the analysis resolution at both spatial and temporal levels (i.e., organ or tissue specific analysis, with more developmental stages). Metabolomic and transcriptome analysis with the assistance of modeling will be of great interest in the future to reveal the network regulating fruit set in response to B:R. Moreover, future investigations will be needed to verify the effect of B:R on fruit set of sweet pepper in a long-term cultivation. Verifying this effect in other crops is also needed, considering the production of many other crop

species (e.g., melons, apples, citrus) also suffers from low fruit set.

5. Conclusions

Sweet pepper plants grown under blue:red light ratio (B:R) of 9:1 showed a substantially lower fruit set compared to those under B:R of 1:10, 1:3 or 1:1. The reduction in fruit set at B:R of 9:1 was associated with a low starch accumulation in the flowers at anthesis and 7 days after anthesis, where the abortion was mostly observed between 9 and 16 days after anthesis. The reduced fruit set and starch content matched with the reduced phytochrome photostationary state (PSS) at B:R of 9:1, where the PSS among the other three B:R was barely changed. Thus, we speculate that these responses might be controlled by phytochromes. The reduced starch content at B:R of 9:1 in flowers seemed to relate to a lower source strength and a drop in sucrose synthases activity. Moreover, on day 7 after anthesis, flowers at B:R of 9:1 contained a lower auxin, higher salicylic acid, higher cytokinin *c*Z and *c*ZR concentration compared to the other B:R. Compared to at anthesis, abscisic acid and ethylene emission on day 7 after anthesis was not reduced at B:R of 9:1 as in the lower B:R. We suggest the reduced floral starch content and the changes in hormonal balance both play a role in reducing fruit set at high B:R of 9:1.

CRediT authorship contribution statement

Ep Heuvelink: Writing – review & editing, Supervision, Resources, Methodology, Data curation, Conceptualization. **Valentina Dalla Villa:** Methodology, Investigation, Conceptualization. **Sijia Chen:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paul Kusuma:** Writing – review & editing, Conceptualization. **Wouter Kohlen:** Methodology, Investigation. **Leo F.M. Marcelis:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Remko Offringa:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ep Heuvelink reports financial support was provided by Fluence LED. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Sijia Chen thanks the China Scholarship Council (CSC) for awarding her a fellowship to conduct this work. We would like to thank Fluence (Austin, Texas, U.S.) for their financial support for the research, as well as Theoharis Ouzounis, and Sebastian Olschowski from Fluence for their interest and discussions. Valentina Dalla Villa thanks Erasmus+ student exchange program for providing the opportunity and scholarship to support her study at Wageningen University. We appreciate help from Zhiming Liu and QianXixi Min for conducting part of the morphological measurements. We thank Maxence Paillart, Arjen van de Peppel, Amber van Seters, Kerstin Gühl, Wannida Sae-Tang, and Naixin Zhang for assisting with lab analyses; Gerrit Stunnenberg and Chris van Asselt for technical support during crop cultivation.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2024.105850.

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