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Research Article

Reproductive Success in Black Pepper before and After Emergence of Inflorescence

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ABSTRACT

Little is known about inflorescence induction and formation cues in the tropical, perennial woody climbing plants. In order to understanding the relationships between photosynthetic distribution in source leaves and inflorescence transition. Different light intensities were imposed on black pepper plants in the field from juvenile to adult phases and verification experiments were carried out on cuttings in the laboratory. Therefore, rely on exogenous measurements to regulate endogenous sugar contents for initiating reproduction success. The dynamic change in photosynthesis, sugar contents and the activity of the enzymes involved in leaves before and after emergence of inflorescence were compared. Pepper source leaves showed quantitative differences in photosynthate distribution in inflorescence induction and formation, before emergence of inflorescence with a marked increase in starch, accompanied by a decrease in sucrose, sucrose-starch ratio, SPS and SS activity from juvenile to adult phase. However, the reverse was true after emergence of inflorescence. Similarly, source leaves carbohydrate allocation in cuttings is typified by a lower sucrose-starch ratio before emergence of inflorescence. Collectively these data point towards the sucrose-starch ratio change for the inflorescence transition in perennial woody climbing plants.

Keywords: Black pepper; Inflorescence; Source leaves; Enzyme activity; Phase transition; Photosynthate distribution

INTRODUCTION

Flower induction in plants is regulated by multiple external and endogenous signals to ensure that flowering occurs at the appropriate time [1]. The coordination between vegetative growth and transition to flowering is critical for reproduction success [2]. Floral inductive cues are originated in mature leaves and mobile signals travel long distances to activate floral identity genes and initiate inflorescence development. Inflorescence induction and suppression in response to environmental signals such as photoperiod and light intensity, has been described in staple crops [3,4]. However, the endogenous inflorescence induction mechanisms which involve physiological readiness to allocate photosynthates in source leaves for floral organ formation in the perennial woody climbing plants such as black pepper (*Piper Nigrum L.*), is less well understood. Black pepper is a perennial plant. The pepper inflorescence is a catkin-like fleshy inflorescence. It arises at the node opposite the leaf on a lateral plagiotropic branch [5]. The lateral branch has sympodial mode of growth, the apical bud develops into the inflorescence and the growth continued by the activity of the axillary bud [6]. In the early development, pepper inflorescence is covered by a leaf- like structure called the prophyll [5]. Emergence of inflorescence from prohyll in black pepper could be regarded as equivalent to the term 'heading' in cereal crops. Pepper plants raised from seeds have a long period of more than three years of vegetative phase and emergence of inflorescence from the prophyll. For plants derived from cuttings emergence of inflorescence is about 1 month after planting in Hainan [7].

Once flowered, pepper plants maintain perpetual flowering habit throughout their lifetimes. This means after the pepper plants there is no distinct period of vegetative growth and reproductive growth. Thus the reproductive growth can compete with vegetative growth for nutrients.

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The common practice of plucking off inflorescence demands a substantial amount of labor force [8]. It has been reported that shading could regulate pepper leaves photosynthesis and in turn controlled inflorescence quantity thus, photosynthates regulate inflorescence induction and formation strategies can be expected to study.

In black pepper, a phenomenon of inflorescence abscission has been observed. Although the shedding process is natural and allows the plant to adjust its reproductive output, this phenomenon can cause important yield losses [9]. Moreover, inflorescence abscission is plant and stress dependent. In rice, control of spike induction and formation is the critical step for successful reproduction [4]. Sugars serve as the plant's energy components; therefore control energy status is the key to survival [10]. Sugars, such as sucrose and starch play important roles in flower induction in response to environmental stresses [11-15]. This connection between sugars metabolism and the floral transition is interesting, especially in physiological studies that link floral induction to carbohydrate changes in mature leaves [16,17]. Recently, in Arabidopsis, characterizations of mutants suggest a link to the regulation of sucrose and starch metabolism [12]. Therefore, these data support the view that the inflorescence induction may involve a coordinated change in carbohydrate metabolism in order to support the process of inflorescence induction and development [2]. Current study on juvenile black pepper vines grown under different shading intensities showed that sugar contents in the leaves affected the inflorescence quantity at different developmental stages. However, it is unknown how coordinated exogenous effects of light intensity and endogenous photosynthesis composition could modify sugars allocation in source leaves for inflorescence induction and formation.

In the developing inflorescence, it was shown that photosynthates are supplied by leaves and leaves are considered as the main source of carbohydrates [18]. The pathway of sugar variation in plant organs is the result of not only complex regulation processes but also reserve mobilization [19,20]. Carbohydrate assimilated in photosynthetic leaves is translocated as sucrose or stored as starch reserves [21]. Generally, the carbohydrate fluctuations depend on the regulation of photosynthates, affecting the related enzyme activities [22,23]. For instance, in young grapevine inflorescences, sucrose represents the main form of circulating sugar during the inflorescence development [24]. Two enzymes are directly involved in sucrose metabolism: (i) the Sucrose Phosphate Synthase (SPS-EC 2.4.1.14), a key regulatory enzyme involved in carbohydrates allocation between sucrose and starch in source leaves is often closely related to the sucrose export rate in source tissues [25,26]. It catalyzes the key step in sucrose synthesis and (ii) the Sucrose Synthase (SS-EC 2.4.1.13) that catalyzes the reversible conversion of fructose and UDP-glucose into sucrose and UDP however, its crucial function in plant metabolism was mainly sucrose breakdown under most physiological conditions and energy provision [27-29]. The light intensity has effects on sucrose-metabolizing enzyme activities in major source leaves of black pepper. Heavy shading markedly decreased the SPS and SS activities. However, the related-enzyme activities in the inflorescence induction and formation are poorly understood.

The objective of this study was to understand the relationships between inflorescence development and sugar metabolism in black pepper. We assayed photosynthesis, carbohydrate metabolism in the source leaves before and after the inflorescences emerged from the prophyll. Our aims are to better characterize carbohydrates metabolism and to try to explain their different ratios of sucrose and starch during the inflorescence development process under optimal conditions. We have studied different carbohydrate metabolism parameters such as photosynthetic capacity, carbohydrate contents and related-enzyme activities of carbohydrate metabolism, before and after inflorescence emergence stage in black pepper. These analyses might elucidate the physiological mechanism of source leaves under shading, to help reproduction success for perennial woody climbing plants.

MATERIALS AND METHODS

Plant materials

Experiment 1: The field experiment was conducted at the pepper field in the Spice and Beverage Research Institute (SBRI) in the southeast Hainan of China (18°72′-18°76′N, 110°19′-110°22′E). This region is characterized as a tropical monsoon climate with an average temperature of 24.6°C, and a maximum light intensity of 1882.0 μmol m^{-2} s^{-1} [8].The annual mean relative humidity is 85%. In this study, black pepper (*Piper Nigrum* cv. Reyin-1) rose from cuttings at the juvenile phase and adult phase were used as the biological materials. Pepper plants raised from cuttings readily flower at about one month after planting and the flowering habit is perpetual. However, there seems to be a main-season and an off-season of flowering. When the environmental factors are favorable such as adequate photo synthetically active radiation, moisture, suitable temperature and after application of fertilizer more inflorescences are produced whereas at off-season i.e. during the dry months and hot season flowering is less. In Hainan, the agronomic practice is to stripe off all inflorescences during the first three year after planting to encourage vegetative growth. At 4th year after planting, inflorescences produced at off-season are also removed. Only the inflorescences produced during the main season are retained to bear fruits. Artificial shading was imposed before and after emergence of inflorescence by placing black polyethylene shading nets with different capacities of shading at 50 cm above the plant canopy, resulting in shading intensities of 30% and 75%. No net covering was taken as the control (CK). There were three replicates in each shading treatment and 11 trees in each replicate. The peppers vines were planted in a north-south row orientation with an inter-row spacing of 2.5 m and an inter-vine spacing of 2.0 m. Three vines were used for collection and recording of numbers of inflorescence every 30 days.

Experiment 2: Indoor light intensity simulation experiment was conducted in the laboratory. In this indoor experiment, cuttings of *P. Nigrum* cv. Reyin-1 were grown in sand and placed

on plant culture shelves (JIUPO-1WSLED-210). Cuttings readily flower at about one month after planting and the flowering habit is perpetual. After cultured for 15 days, four light intensity treatments, i.e.135 $\mu molm^{-2}s^{-1}$, 270 $\mu molm^{-2}s^{-1}$, 405 $\mu molm^{-2}s^{-1}$ and 540 $\mu molm^{-2}s^{-1}$, were imposed. There were four replicates in each light intensity treatment and two cuttings in each replicate.

Measurement of photosynthetic parameters

Photosynthetic rate (Pn) was measured before and after emergence of inflorescence i.e. in the juvenile and adult phases on field-grown pepper plants as well as cuttings on the second leaf away from the inflorescence using a gas-exchange meter (LI-6400, LI-COR, Lincoln, NE, USA). The measurement was carried out between 14:30 h and 16:30 h in three black pepper plants in the field and two cuttings in the laboratory for each treatment.

Leaf carbohydrate content determination

Carbohydrate extraction: 0.25 g source leaves was ground in liquid N_2. The powder was transferred into a centrifuge tube, added with 4 mL of alcohol, and oscillated at 80°C for 30 min and then centrifuged at 8,000 g and 4°C for 20 min (Sigma 3 K-18 K). The supernatant was then transferred into a test tube. The extraction was repeated twice. The three supernatants were combined and diluted to 50 ml for sucrose and starch determination [27].

Leaf sucrose assay

Sucrose analyses were performed using 1 mL of the extract boiled with 200 µL of 2 mol/L sodium hydroxide at 100°C for 10 min. Then 3.5 mL of 30% hydrochloric acid and 0.8 ml of 0.1% resorcinol were added and the reaction was terminated at 80°C for 10 min. Once cooled, the reaction absorbance was measured at 480 nm. Sucrose concentration was calculated according to a sucrose calibration range [30-37].

Leaf starch assay

1 mL of the extract was used to determine starch content. The extract was mixed with 5 ml of anthrone-sulfuric acid and incubated at 100°C for 5 min. Once cooled, the alcohol and water were removed with a centrifugation evaporator. Then 2 ml of deionized water was added, mixed and incubated at 100°C for 15 min and after cooling down, 2 ml of 9.2 mol/l perchloric acid was used to extract for 15 min. The sediment was filtered and the volume of filtrate was adjusted to 25 ml. The sediment was extracted by 2 ml of 4.6 mol/l perchloric acid for 10 min. Next, the filtrate and 6 ml of deionized water were mixed together in a 25 ml volumetric flask. 1 ml mixture and 5 ml of anthrone-sulfuric acid reagent was mixed, and then boiled at 100°C for 5 min. Once cooled, the reaction absorbance was measured at 620 nm [30].

Leaf carbohydrate metabolism enzyme extraction

Frozen leaf tissue (0.5 g) was homogenized with 0.08 g of PVPP in 4 ml of extraction buffer (50 mM HEPES-NaOH, pH 7.5, 20 mM $MgCl_2$, 2 mM EDTA, 2.5 mM DTT, 1 mM PMSF and 0.05% Triton X-100). The homogenate was centrifuged at 4°C and 10,000 g for 20 min. SPS and SS activity analysis SPS activity was assayed using 100 µL of enzyme extract in 150 µL of reaction buffer (50 mM HEPES-NaOH, 10 mM $MgCl_2$, 10 mM F-6-P, and 3 mM UDPG, final pH 7.4). The reaction was initiated by incubating the reaction at 30°C for 30 min. The reaction was terminated at 100°C for 10 min with 50 µL of 2 M NaOH. The sample was then cooled. The reaction was terminated at 80°C 10 min with 875 µL of 30% (w/v) HCl and 250 µL of 0.1% (w/v) resorcinol. A standard curve of sucrose was used to calculate for sucrose content. For the SS activity assay, F-6-P in the reaction buffer was replaced by fructose, and the other reaction steps were the same as those in the SPS activity analysis [27].

Statistical analysis

All experiments data were the means of at least three replicates with standard errors. Histograms and line graphs were developed using GraphPad. Prism. v5.0 (Cabit Co., USA). Statistical analyses were carried out using unpaired two-tailed t-tests at 95% confidence (version 8.2, SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Effects of light intensity on inflorescence formation

Originated in the forest of Western Ghats of India, black pepper is shade tolerant. This makes it possible for an investigation on the regulation of light intensity on inflorescence induction and formation Under different shade intensities, pepper plants grown in the field under 30% shade showed a slight higher inflorescence number as compared to natural sunlight during juvenile phase and adult phase. Under 75% heavy shading, the inflorescence number was markedly reduced as compared to that under natural sunlight and 30% shading in the juvenile phase (Figures 1A and 1B).

A study was conducted to examine the influence of light on the number of inflorescence emerged and shed by exposing pepper cuttings to different light intensities. Under 405 $\mu molm^{-2}s^{-1}$ light intensity, the number of inflorescence emerged is significantly higher and number of inflorescence shed is markedly lower than the other three treatments. The highest inflorescence induction and formation affords a unique system for studying the endogenous reproduction pathway, since the developmental signals to initiate a successful transition are absent in the black pepper. Photosynthetic rate of source leaves before and after emergence to investigate whether the observed higher quantity of inflorescence induction and formation of black pepper might be due to increased photosynthetic rate in source leaves the photosynthetic rate before and after emergence of inflorescence was compared. Data taken from the field-grown plants at juvenile and adult phases and cuttings grown in the laboratory were analyzed. For pepper plants grown in the field, photosynthetic rate (Pn) in the control and the two shading treatments showed a slight decrease after emergence of inflorescence compared to that before emergence of inflorescence at juvenile phase (Figures 2A-2C).

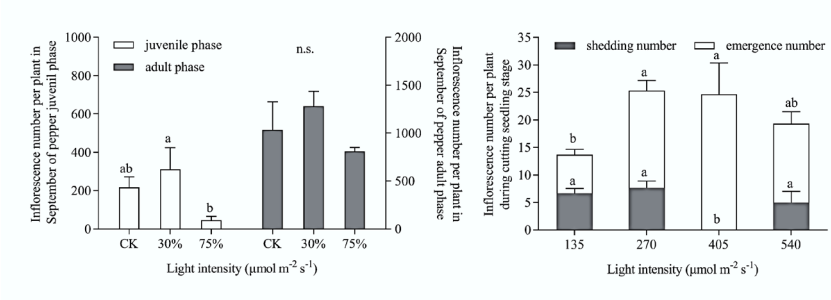

Figure 1: (A): Changes in pepper inflorescence number at juvenile and adult phases of field-grown black pepper plants and cuttings (B): Under different light intensities. Error bars indicate s.e.m (n=3); in the same development phase, columns sharing different letters indicate significant differences according to the ANOVA in SAS (P<0.05).

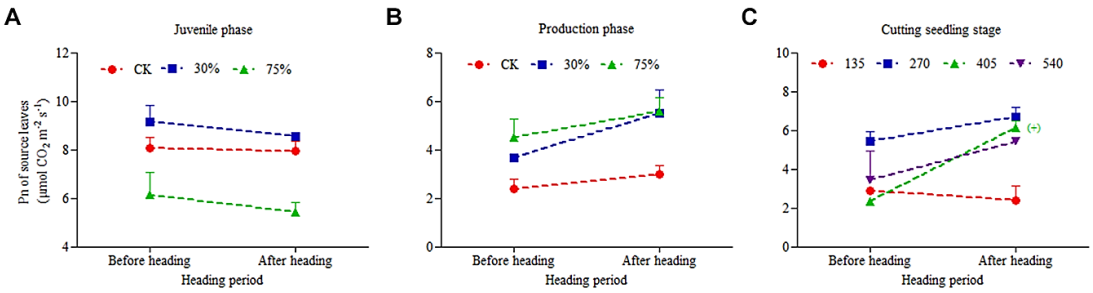

Figure 2: (A-C): Changes in source leaf photosynthetic rate (Pn) at juvenile and adult phases of field-grown black pepper plants and cuttings under different light intensities in the laboratory. Statistical significance of Pn before and after emergence of inflorescence based on a t-test (P<0.05) is indicated with (+). Error bars represent the SD (n=3).

At the adult phase, Pn increased in pepper plants grown under natural sunlight and under the two shading treatments after emergence of inflorescence. In the case of cuttings, Pn increased after emergence of inflorescence. However, as far as the field-grown pepper plants are concerned, the difference in Pn before and after emergence of inflorescence either in the juvenile or adult phase are not significant statistically. Among the different light intensities, cuttings increased in all light intensities as well as in the control. However, the increase in Pn was not statistically significant except that under 405 μmolm^{-2}s^{-1} light intensity. Then, since changes in the photosynthetic rate were observed before and after emergence of inflorescence, the sucrose and starch levels were examined to assess possible changes in the carbohydrate allocation in source leaves. Ratio of sucrose and starch content in leaves before and after emergence of inflorescence were compared to delineate whether similar changes in the levels of sucrose and starch observed in juvenile phase and adult phase in field-grown plants as well as cuttings grown in the laboratory in pepper. For the field experiments, pepper was grown under full sun light (CK) and 30% as well as 75% shading intensities. It was found that pepper inflorescence was induced, starch increase and sucrose decrease in plants growing under natural sunlight and 30% shade whereas plants grown fewer than 75% shade recorded significant increase in the sucrose level before emergence of inflorescence in juvenile phase (Figures 3A-3C).

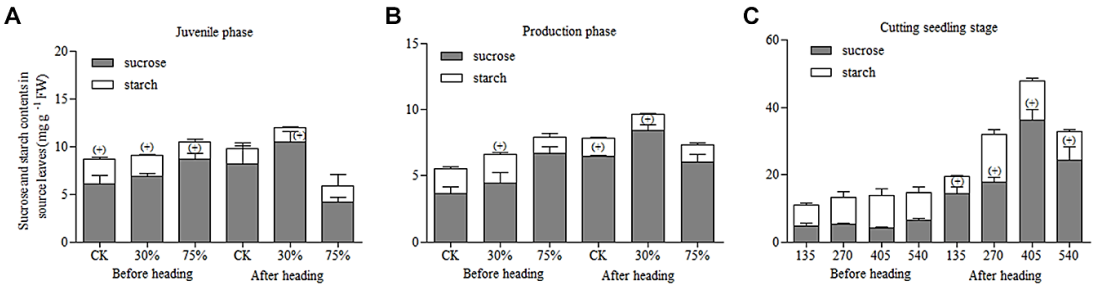

Figure 3: (A-C): Changes in source leaf sucrose and starch contents at juvenile and adult phases in field-grown black plants and cuttings under different light intensities in the laboratory. Statistical significance of sucrose and starch contents before and after emergence of inflorescence based on a t-test (P<0.05) is indicated with (+). Error bars represent the SD (n=3).

Similar trend was recorded in adult phase especially for plants grown natural sunlight and under 30% shades. Together, these data show that the increase in the number of inflorescence can be attributed to apparent starch accumulation which was detected in pepper leaves before emergence of inflorescence. For the laboratory experiment, it was found that, no remarkable accumulation of starch in cuttings before emergence of inflorescence. Therefore, changes in starch levels are associated with emergence of inflorescence in juvenile and adult phases but not in cuttings. After the emergence of inflorescence, retention of inflorescence is also important for pepper yield improvement. With respect to the changes in sucrose content in the leaves of cuttings, it was found that sucrose level increased in cuttings growing under 135 to 540 $\mu molm^{-2}s^{-1}$ light intensities and is particularly obvious in those growing under 135 and 270 $\mu molm^{-2}s^{-1}$ light intensities after emergence of inflorescence. Therefore, increase in the leaves sucrose levels after emergence of inflorescence is important for inflorescence formation. Although under 135 and 270 $\mu molm^{-2}s^{-1}$ light intensities, sucrose levels increased significantly after emergence of inflorescence, the abscission of inflorescence in cuttings growing under these two light intensities is marked. So there could be some other physiological changes associated with the inflorescence abscission. Sucrose-starch ratio of source leaves before and after emergence of inflorescence. The relative ratio of sucrose to starch was also examined in pepper leaves at different development stages. Under natural sun light (CK) and 30% shade, pepper at both the juvenile and adult phases the sucrose-starch ratio before emergence of inflorescence is less than 50% of that after the emergence of inflorescence (Figures 4A and 4B).

However, for plants growing under 75% the reverse is true. The elevated inflorescence number under CK and 30% shading intensity conditions can be attributed to apparent decrease of sucrose-starch ratio before emergence of inflorescence. It is also intriguing that, leaves of cuttings grown under 135 $\mu molm^{-2}s^{-1}$ light intensity although also recorded lower sucrose-starch ratios before emergence of inflorescence as compared to that after emergence of inflorescence, the inflorescence number was not significantly induced. After emergence of inflorescence, the 4.5-fold increase of sucrose- starch ratio the leaves of cuttings grown fewer than 405 $\mu molm^{-2}s^{-1}$ light intensity may contribute to the inflorescence formation. The increase of sucrose-starch ratio being much lower than that recorded for plants grown under other light intensities could be one of the main reasons for the highest number of inflorescence shedding in plants growing under 270 $\mu molm^{-2}s^{-1}$ light intensity (Figure 4C).

Therefore, the level of the sucrose-starch ratio before and after emergence of inflorescence could influence inflorescence formation. Activities of sugar metabolizing enzymes in pepper source leaves. Sucrose metabolizing enzyme activities in source leaves at different developmental phases were studied. Comparing to the before emergence of inflorescence, there was an increase of 3.6 to 12.3-fold of SPS activity in leaves of pepper grown under different light intensities after emergence of inflorescence during juvenile phase (Figure 5A).

SPS activity increased 2.0-fold after emergence of inflorescence in 75% shading intensity during adult phase. However, no significant difference (P>0.05) in SPS activity was observed in CK and 30% shading intensity before and after emergence of inflorescence during adult phases (Figure 5B).

Moreover, no significant difference was observed in SPS activity before and after emergence of inflorescence in cuttings (P>0.05) growing under all the four light intensities. In the field-grown pepper plants, the levels of SS activity in source leaves increased 3.6 to 14.7-fold after emergence of inflorescence as compared to before emergence of inflorescence in the juvenile phase. However, no significant difference was observed before and after emergence of inflorescence in the adult phase in the field-grown pepper plants as well as in the cuttings (Figures 5C-5F).

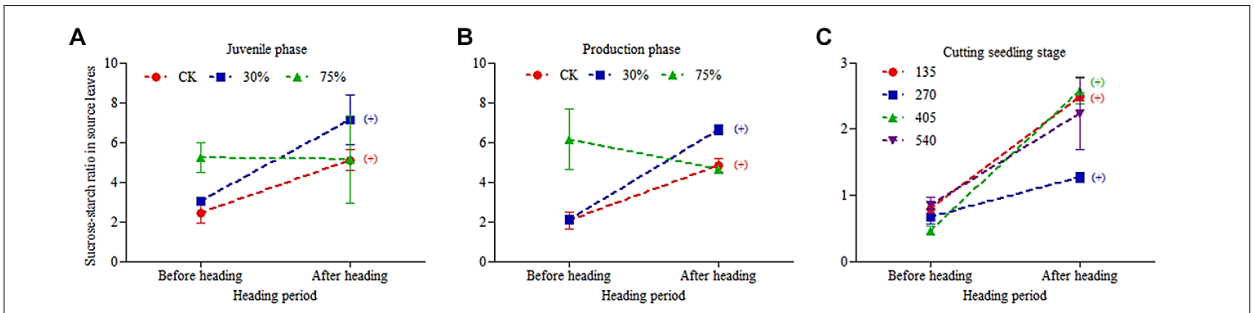

Figure 4: (A-C): Changes in source leaf sucrose-starch ratio at juvenile, adult phases in field-grown black pepper plants and cuttings under different light intensities in the laboratory. Statistical significance of the sucrose-starch ratios before and after emergence of inflorescence at each development phase based on a t-test (P<0.05) is indicated with (+). Error bars represent the SD (n=3).

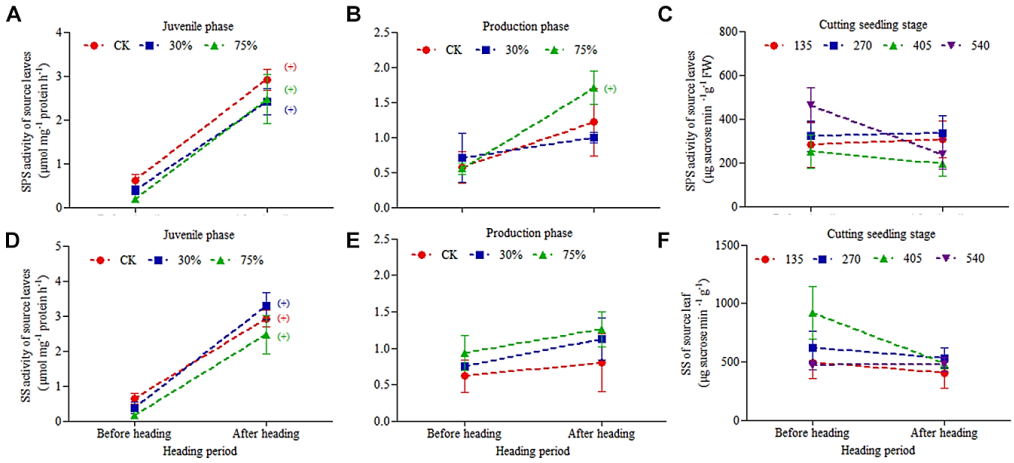

Figure 5: (A-F): Changes in source leaf sugar metabolizing enzyme activities at juvenile and adult phases in field-grown black pepper plants and cuttings under different light intensities. Statistical significance of the enzyme activities before and after emergence of inflorescence based on a t-test (P<0.05) is indicated with (+). Error bars represent the SD (n=3).

CONCLUSION

In this study, the decrease of sucrose-starch ratio before and increase after emergence of inflorescence is the key regulator for the success of inflorescence formation. SPS activity in source leaves of three stages had different correlations with ratio of sucrose and starch. There was a significantly positive correlation between SPS activity and sucrose-starch ratio (R=0.476, n=18, P<0.05) in source leaves during juvenile phase. There was no significant correlation between SPS activity and sucrose-starch ratio in source leaves in the adult phase and in the cuttings. The ratio of sucrose and starch was significantly affected by Pn (R=0.628, n=18, P<0.01) in source leaves at adult phase. The increase in the levels of sucrose and starch were significantly correlated with the increase of Pn (R=0.553 for sucrose, R=0.683 for starch, n=24, P<0.01) in source leaves of cuttings before emergence of inflorescence, but had only slight effects on sucrose-starch ratio. SS (EC 2.4.1.13) is another key regulator of sucrose synthesis. There was a significant correlation between SS activity and sucrose-starch ratio (R=0.531, n=18, P<0.05) in source leaves at the juvenile phase. However, the SS activity had no significant correlation with ratio of sucrose and starch in source leaves during adult phase and cuttings. These results indicated that SPS activity, SS activity and photosynthesis could probably regulate the sucrose and starch levels in source leaves, and higher photosynthetic rate as well as higher activity of SPS and SS could improve formation of inflorescence in pepper.

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Not applicable

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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