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Fructose Regulation of Stomatal Movement and its Effects on the Photosynthetic System in *Vicia faba* L.

Duoyi Wang[1#], Rui Li[2#], Yali Zhang[3], Jing Gao[2], Nan Wang[1], Yonggang Yan[2], Gang Zhang[2]

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ABSTRACT

Background: *Vicia faba* L. is a significant grain legume that is rich in protein and nutrients. It is widely cultivated for human consumption and animal feed in temperate regions. As a signaling molecule, fructose is involved in many metabolic and developmental processes. The objective of this study was to demonstrate whether fructose may increase reactive oxygen species (ROS) production in guard cells inducing stomatal closure and examine whether the exogenous application of fructose may regulate photosynthesis and chlorophyll fluorescence characteristics.

Methods: After approximately 4 weeks of sowing the treated broad bean seeds, the fully inflated leaves were used for experimental data collection including stomatal movement, gas exchange, chlorophyll fluorescence and ROS production in guard cells.

Result: Fructose reduced stomatal conductance (*Gs*), transpiration rate (*Tr*) and stomatal aperture of intact leaves, indicating that fructose can trigger stomatal closure in a dose- and time-dependent manner in the epidermal peels of the broad bean plant (*Vicia faba* L.). Meanwhile, fructose increased the photochemical efficiency, photosynthetic rate (*Pn*) and water use efficiency (WUE). Fructose-induced stomatal closure was associated with nitric oxide (NO), calcium ion (Ca^{2+}), aquaporin and ROS.

Key words: Hydrogen peroxide, Nitric oxide, Photosynthesis, Stomatal movement, Sugar.

INTRODUCTION

Broad bean (*Vicia faba* L.) belongs to the legume family and are known for their high levels of protein, nutrient and bioactive compounds that are commonly used in food and feed (Dewangan *et al*., 2022). Sugar not only provides energy for plant growth, but also plays a role as a signaling molecule in the life cycle of plants (Rolland *et al*., 2006). ROS and NO are the core signals that regulate stomatal movement (Nazareno and Hernandez, 2017). Previous studies have proved that glucose and mannose can promote stomatal closure in broad beans through hydrogen peroxide (H_2O_2), NO and Ca^{2+}-based signaling (Li *et al*., 2018). Sucrose can reduce stomatal opening and is easily decomposed by sucrase into glucose and fructose (Kelly *et al*., 2013). Studies have shown that fructose can also inhibit stomatal opening (Dittrich and Mayer, 1978), but details of the signaling mechanism and regulatory pathways are not well-understood.

Both the external environment and internal signaling molecules can regulate stomatal movement. NO can stimulate the release of Ca^{2+} from calcium pools, resulting in an increase in free cytoplasmic Ca^{2+} and inhibition of inward rectifying K^+ channels. As a result, the plasma membrane localized anion channels in guard cells are activated, consequently leading to an anionic outflow. Simultaneously, it promotes the synthesis of phospholipid acid and enhances the ROS levels, causing stomatal closure (Sun *et al*., 2019). However, it is not known whether fructose induces ROS production in guard cells and information regarding its mechanism of production is also lacking.

Exogenous sugar can increase the photosynthesis-based substrate supply in plants, help the plants maintain a stable photosynthetic performance, enhance their tolerance under stress and reduce damage to the photosynthetic machinery (Sun *et al*., 2019). Previous studies have shown that glucose and sucrose can reduce stress-induced damage and inhibition and increase *Pn* and photosystem II (PSII)-based activity. PSII is highly susceptible to stress and is closely related to various photosynthesis-related physiological processes (Zhang *et al*., 2020a).

[1]State Key Laboratory of Research and Development of Characteristic Qin Medicine Resources (Cultivation), Co-construction Collaborative Innovation Center for Chinese Medicine Resources Industrialization by Shaanxi and Education Ministry, Shaanxi University of Chinese Medicine, 712083, Xianyang, China.
[2]College of Pharmacy, Shaanxi University of Chinese Medicine, 712046, Xianyang, China.
[3]Health Department, Shaanxi Fashion Engineering University, 712046, Xianyang, China.
[#]These authors contribute equally to this work.

Corresponding Author: Nan Wang, State Key Laboratory of Research and Development of Characteristic Qin Medicine Resources (Cultivation), Co-construction Collaborative Innovation Center for Chinese Medicine Resources Industrialization by Shaanxi and Education Ministry, Shaanxi University of Chinese Medicine, 712083, Xianyang, China. Email: wangnan@sntcm.edu.cn

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In this experiment, our objectives were to (1) demonstrate whether fructose may increase ROS production in guard cells inducing stomatal closure; (2) examine whether the exogenous application of fructose may regulate photosynthesis and chlorophyll fluorescence characteristics.

MATERIALS AND METHODS

Plant materials and growth conditions

The seeds of the broad bean (*Vicia faba* L. cv. Da qing pi) were purchased from Shanghai NongLe Cultivation Co., Ltd., China. The experiment began in September 2022 and was carried out in the plant cultivation room and laboratories of Shaanxi University of Chinese Medicine. The seeds were sterilized in 75% ethanol for 30 min and then washed with water 5 to 6 times. Sterilized seeds were soaked in water for 48 h and then grown in a growth chamber at temperatures ranging from 20-25°C, under conditions of 70% relative humidity (RH), photosynthetically active radiation (PAR) of 300 μmol m^{-2} s^{-1} and a photoperiod of 14 h light/10 h dark, with daily watering schedule. Approximately 4 weeks after seeding, the completely expanded leaves were cut and the abaxial epidermis was peeled off.

Chemical reagents

Molecular probe 2',7' dichlorofiuorescin diacetate (H_2DCF-DA from Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO from Amresco, Solon, OH, USA) to produce a stock solution, which was aliquoted. Diphenylene iodonium chloride (DPI), salicylhydroxamic acid (SHAM), reduced glutathione (GSH), 2 (4 carboxyphenyl) 4, 4, 5, 5 tetramethylimidazoline 1 oxyl 3 oxide (c-PTIO), catalase (CAT, bovine liver), NG-nitro-L-arginine methyl ester (L-NAME) and 2 (N-morpholino) ethanesulfonic acid (MES) were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Unless stated otherwise, the remaining chemicals were bought from companies based in China. All the chemicals used in this study were of the highest analytical grade available.

Stomatal bioassay

Stomatal bioassay experiments were performed as described with slight modifications. The details can be obtained from Gao *et al*. (2013). The strips were soaked in MES-KCl buffer solution (10 mM MES, 50 mM KCl, 100 mM KOH, pH= 6.1) for 2 h to induce stomatal opening and then transferred to fructose solution having different concentrations (0, 100, 125, 135 and 150 mM, respectively) for 30 min incubation in light. The stomatal aperture sizes were determined using digital images captured by a microscope (BX53, Olympus, Tokyo, Japan).

Measurement of ROS production in guard cells

The details can be obtained from Gao *et al*. (2013). The intensity of fluorescence was quantified using the Image Pro Plus 6.0 software (Media Cybernetics, Silver Springs, MD, USA). In each treatment, we recorded the measurements from three epidermal strips originating from different source plants. The experiment was repeated three times. The data represent the means of 120 measurements ± SE.

Gas exchange measurements

The 4-week-old plants were sprayed with 125 mM fructose dissolved in water and the controls were sprayed with water. At 3 h, 24 h and 48 h after treatment, leaf gas exchange was measured using a portable photosynthetic apparatus CIRAS-3 (PP Systems, UK) (Li *et al*., 2018). The carboxylation efficiency (CE) was calculated as *Pn*/*Ci* (Teramura *et al*., 1990). The stomatal limit value LS was calculated as 1 *Pn*/*ca*. The experiment was repeated four times (n= 4).

Chlorophyll fluorescence measurements

The chlorophyll fluorescence parameters were determined using a Pocket PEA fluorimeter (Plant Efficiency Analyzer PE 321JL, Hansatech Instruments Ltd., King's Lynn Norfolk, UK) (Guha *et al*., 2013). The 4-week-old plants were sprayed with 120 mM fructose dissolved in water and the controls were sprayed with water. Gas exchange estimations were measured on the same leaves for which the chlorophyll fluorescence induction (OJIP) parameters were conducted. Leaves used for chlorophyll fluorescence assay were collected at 3 h, 24 h and 48 h after treatment. After full dark adaptation for 30 min, the indices were determined. Leaves at the same stages of photosynthesis were selected for the estimation. The chlorophyll fluorescence parameters were: average absorbed photon flux per PSII reaction center (ABS/RC), dissipated energy flux per PSII (DI_O/RC), maximum trapped excitation flux per PSII (TR_O/RC), electron transport flux from Q_A to Q_B (ET_O/RC), electron transport flux until PSI acceptors per PSII (RE_O/RC), performance index for energy conservation from photons absorbed by PSII antenna to the reduction of PSI acceptors (PI_{total}), performance index for energy conservation from photons absorbed by PSII antenna to the reduction of Q_B (PI_{ABS}), maximum quantum yield of primary PSII photochemistry (φ_{PO}), probability of electron transport beyond Q_A^- (Ψ_{EO}), quantum yield of the electron transport flux from Q_A to Q_B (φ_{EO}), efficiency with which an electron from Q_B is transferred to PSI acceptors (δ_{RO}) and quantum yield of the electron transport flux till the electron reaches the PSI electron acceptors (φ_{RO}), respectively. The experiment was repeated six times (n = 6).

Statistical analysis

Standard error (SE) for each treatment was calculated. Differences among treatments were compared using least significant difference (LSD, $P<0.05$) test. All statistical analyses were performed using the SPSS 26.0 package (SPSS, Chicago, USA).

RESULTS AND DISCUSSION

Fructose-induced stomatal closure in epidermal strips

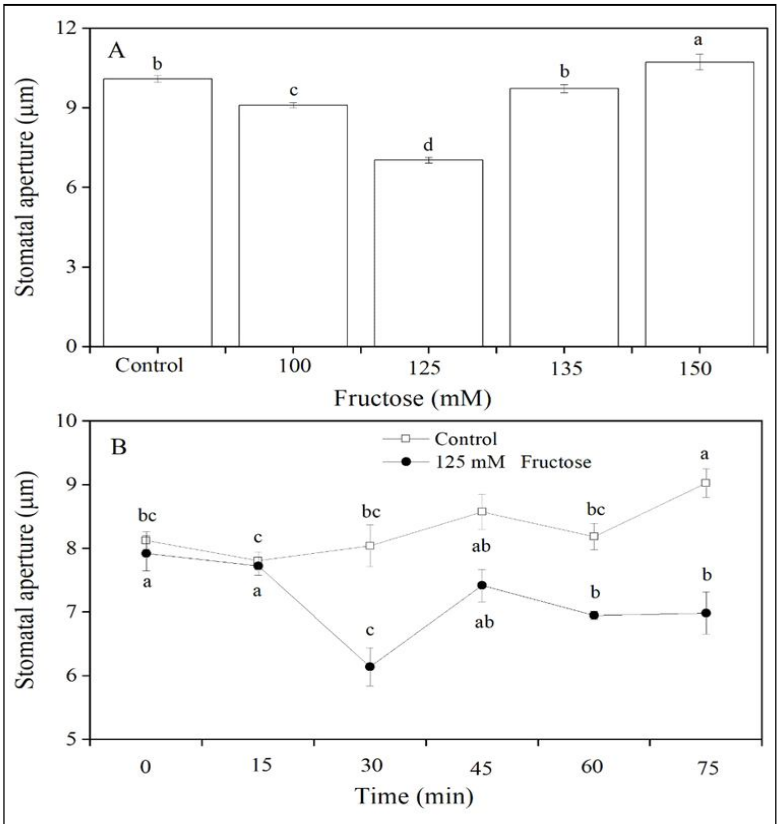
Fructose is a soluble sugar found in higher plants, which has high superoxide scavenging capacity (Bogdanović *et al*.,

2008). In this study, after full illumination, the stomata opened and their apertures increased to approximately 10.09 μm in the control group (Fig 1A). After the application of 100 mM, 125 mM and 135 mM fructose, respectively, for 30 min under light, stomatal apertures were reduced by 9.79% (P<0.01), 30.27% (P<0.01) and 3.58% (P>0.05), respectively, compared to the control (Fig 1A). However, stomatal aperture increased by 6.28% (P<0.05) under 150 mM fructose treatment (Fig 1A). With the increase of fructose concentration, stomatal opening decreased first and then increased. The results showed that fructose-induced stomatal closure in the epidermal strips of the broad bean was concentration-dependent. The effects of fructose at higher concentrations (135 and 150 mM) were less, which rules out an osmotic role of sugars in mediating stomatal closure (Li *et al*., 2016). The time course of stomatal closure in broad beans is shown in Fig 1B. Stomatal aperture decreased rapidly with the treatment time and reached the minimum value at 30 min, showing a significant reduction by 23.62% (P<0.05) compared with that in the control. Our results suggested that fructose induce stomatal closure in a dose- and time-dependent manner.

H_2O_2 and NO participate in fructose induced stomatal closure in guard cells

H_2O_2 exists in cells and tissues as a molecule form of ROS (Govindaraj *et al*., 2017). As shown in Fig 2, when compared to the 125 mM fructose treatment, the CAT, DPI, GSH and SHAM-based treatments increased stomatal aperture by 66.64%, 46.90%, 27.19% and 27.03%, respectively. The implication is that H_2O_2 is involved in fructose-induced stomatal closure. In this study, CAT was found to completely reverse fructose-induced stomatal closure. CAT is membrane-impermeable with efficient catalytic and regulatory properties and can degrade H_2O_2 into water and oxygen (Sharma and Ahmad, 2014), implying that sugar-induced ROS production occurs exclusively outside the plasma membrane of guard cells (Li *et al*., 2016). Additionally, ROS production can be inhibited by both DPI and SHAM to similar extents, which suggested that both NADPH oxidase and peroxidase activity can be responsible for fructose-triggered oxidative burst (Hemetsberger *et al*., 2012).

NO is important components of complex signals transmitted during the process of stomatal movement



(A) Dosage effect of fructose-induced stomatal closure. (B) Time-response curve of stomatal closure induced by 125 mM fructose. Stomatal apertures were assessed every 30 min. Means without a common letter are significantly different among treatments as determined by ANOVA. (LSD test, P<0.05).

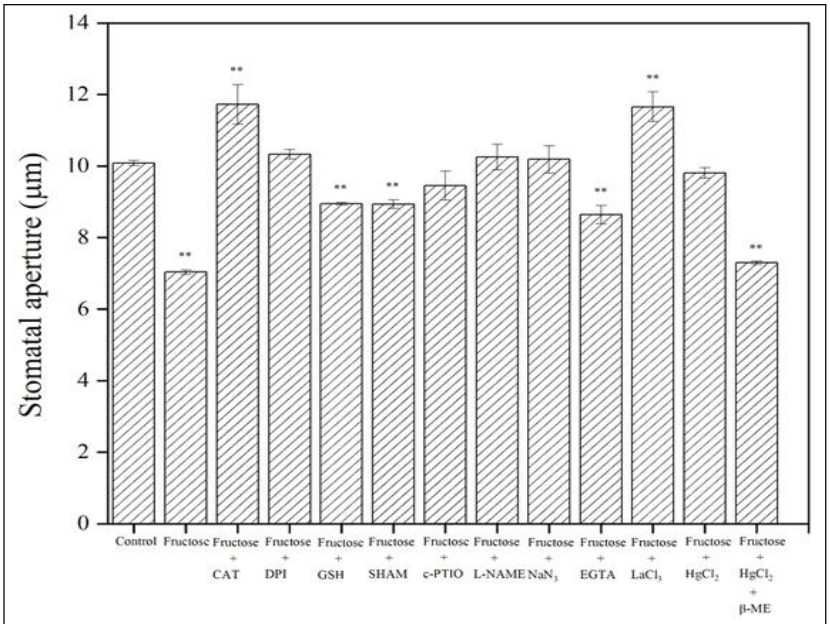
Fig 1: Fructose-induced stomatal closure in epidermal peels of broad bean.

(Ajaykumar *et al*., 2023; Li *et al*., 2018). Meanwhile, NO protects plants from damage caused by oxidative stress (Dadasoglu *et al*., 2021). Fig 2 also showed that fructose-induced stomatal closure can be inhibited by 10 μM NO scavenger (c-PTIO), 25 μM NOS inhibitor (L-NAME) and 25 μM NR inhibitor (NaN_3), with stomatal opening being restored up to 93.99%, 101.92% and 101.28%, respectively, of that in the control group. These data suggested that fructose-mediated NO production is generated *via* NOS and NR-mediated mechanisms (Melotto *et al*., 2006).

The fluorescence intensity of H_2DCF-DA in guard cells of broad beans increased significantly by 61.60% (P<0.05) and 126.94% (P<0.05) after 100 mM and 125 mM fructose treatment, respectively, when compared to the control, while there was no significant change when treated with other concentrations of fructose (Fig 3B; P>0.05). In contrast to the 125 mM fructose treatment, H_2DCF-DA fluorescence intensity of the guard cells of broad beans decreased significantly by 75.20% (P<0.05), 65.48% (P<0.05), 45.62% (P<0.05) and 45.22% (P<0.05), respectively, after CAT, DPI, GSH and SHAM treatment (Fig 3A). These results indicate that fructose can induce an increase in the H_2O_2 content in the guard cells and the H_2O_2 catalyzed by NADPH oxidase and peroxidase is involved in fructose-induced stomatal closure. This observation is in agreement with the stomatal response noted in Fig 2, further suggesting that fructose-induced stomatal closure is mainly mediated by ROS *via* DPI-sensitive plasma membrane NADPH oxidases and not *via* SHAM-sensitive peroxidases.

Ca^{2+} and aquaporin are involved in fructose induced stomatal closure

2 mM calcium chelating agent ethylene glycol tetra acetic acid (EGTA) and 1 mM calcium antagonist lanthanum chloride ($LaCl_3$) significantly inhibited fructose-induced stomatal closure (P<0.05; Fig 2). Treatment regimens with EGTA and $LaCl_3$, respectively, after treatment with 125 mM fructose, allowed stomatal aperture recovery to 85.70% (P<0.05) and 115.54% (P<0.05) of that in the control. Fructose-induced stomatal closure was inhibited by 50 μM water channel blocker mercuric chloride ($HgCl_2$), with the stomatal aperture of 125 mM fructose-treated epidermis being restored to 97.26% of that in the control. β-Thiol (β-ME) prevented binding of $HgCl_2$ to aquaporin. The effect of $HgCl_2$ on stomatal movement was neutralized by β-ME (Fig 2). These results suggest that Ca^{2+} and aquaporin are involved in fructose induced stomatal closure.



Effects of CAT, DPI, GSH, SHAM, c-PTIO, L-NAME, NaN_3, EGTA, $LaCl_3$ and $HgCl_2$ on fructose-induced stomatal closure. Epidermal peels of broad bean pre-incubated for 2 h in opening buffer under light were treated with or without 20 μm DPI, 2 mM SHAM, 2 mM GSH and 100 U ml^{-1} CAT, 10 μM c-PTIO, 25 μM L-NAME and 25 μM NaN_3, 2 mM EGTA, 1 mM $LaCl_3$, 50 μM $HgCl_2$ and 50 μM $HgCl_2$ containing 5 mM β-ME for 30 min, then the stomatal apertures were determined after applications of 125 mM fructose, respectively, for further 30 min. ** and * indicate significant difference at the P<0.01 and P<0.05 between the treated and control plants, respectively (LSD test, P < 0.05).

Fig 2: Involvement of ROS and NO production, Ca^{2+} and water channels in fructose-triggered stomatal closure.

Effects of fructose on stomatal opening and gas exchange parameters

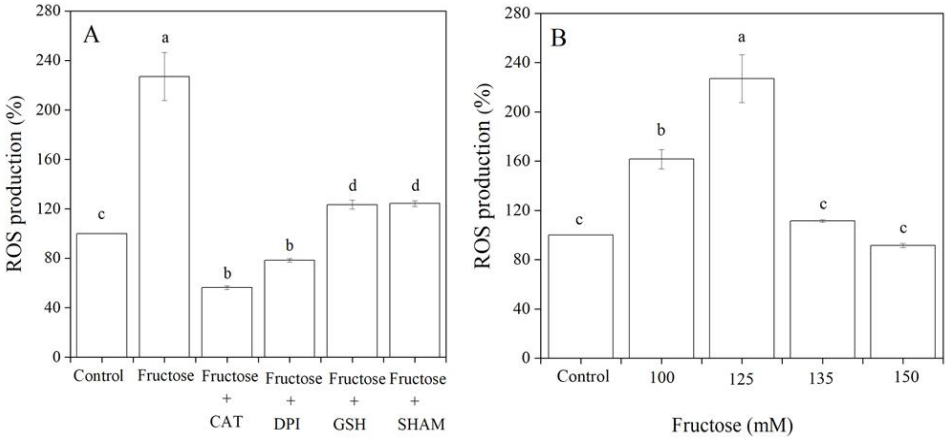
As noted in Fig 4A, *Pn* increased by 20% (P<0.001) and 18.11% (P<0.001), respectively, at 3 h and 48 h after fructose treatment compared to that in the control. The *Ci* at 3 h after fructose treatment increased by 11.55% (P<0.001) compared to the control (Fig 4D). Compared with the control, *Gs* and *Tr* increased at 3 h and 48 h, respectively, after fructose treatment and decreased significantly at 24 h after fructose treatment (Fig 4B, C). Meanwhile, exogenous application of fructose significantly increased the carboxylation rate (CE) at 24 and 48 h (Fig 4E) (P<0.001). LS reached its maximum value at 24 h after fructose treatment (Fig 4F) (P<0.001). The saturated vapor pressure difference (VPD) was significantly lower than that of the control at 48 h after fructose treatment (P<0.001) and there was no significant difference at other time points (Fig 4G). WUE was significantly increased at 24 h after fructose treatment (P<0.001) and there was no significant difference at other time points (Fig 4H). Compared to the control, stomatal aperture decreased by 22.77% (P<0.001) and 5.27% (P<0.05) at 3 h and 24 h, respectively, after spraying of fructose (Fig 4I). Therefore, *Pn* did not differ significantly between the treated group and the control at 24 h.

Effects of fructose on rapid chlorophyll fluorescence induction kinetic curve

The rapid chlorophyll fluorescence induction kinetic curve of plants contains a lot of information about the initial photochemical reaction of the PSII reaction center. The non-destructive analysis of polyphasic fast chlorophyll *a* fluorescence (ChlF) has been widely used to reflect a plant's photosynthetic performance (Živčák *et al*., 2015; Hajihashemi *et al*., 2020). Exogenous fructose reduced the rapid-induction curve between J and I phase, but the difference was not significant. OJIP from fructose-treated plants gave a lower fluorescence yield at J-I phase (Fig 5).

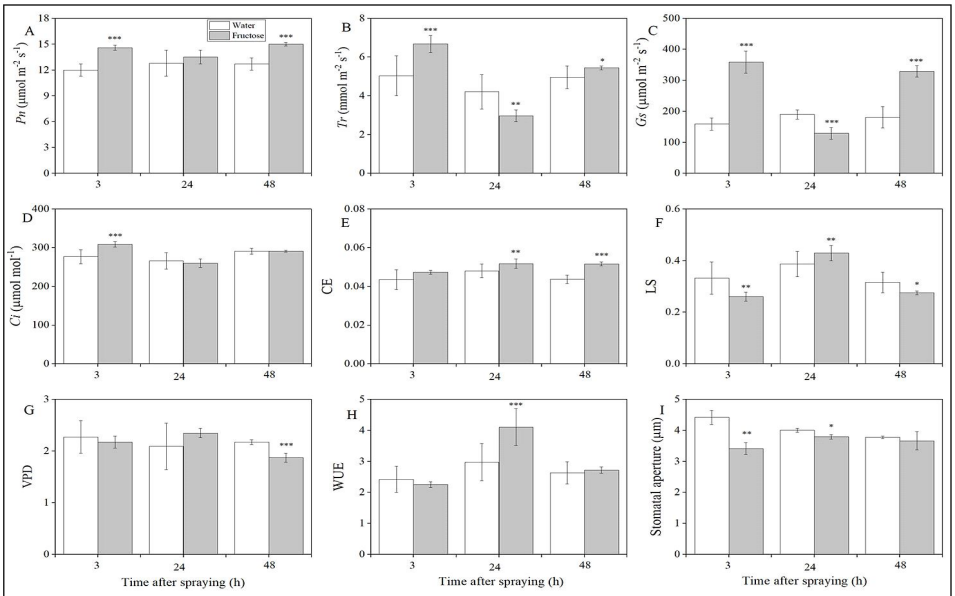
Effects of fructose on PSII reaction center activity

Table 1 shows that the light energy absorbed by the chloroplast unit reaction center (ABS/RC) of broad bean leaves after exogenous fructose treatment decreased by 13.9% (P<0.05) and 14.0% (P<0.05) at 3 h and 48 h, respectively, compared with the control. ABS/RC, TR_O/RC, ET_O/RC and DI_O/RC on PSII receptor side belong to the specific activity parameters of PSII in unit reaction center and the decrease in ABS/RC, TR_O/RC and ET_O/RC indicates that the electron transport capacity has increased (Papazi *et al*., 2008). The maximum photochemical efficiency (φ_{PO}) and the energy dissipated per unit reaction center (DI_O/RC and RE_O/RC) in the fructose treated groups were not statistically different compared with the control group (P>0.05). The light energy captured per unit reaction center (TR_O/RC) and the energy used for electron transport per unit reaction center (ET_O/RC) were significantly reduced at 3 h and 24 h, respectively, after fructose treatment. The probability of electron transport outside QA (ψ_{EO}), photosynthetic performance index (PI_{ABS}: the energy conservation from photons absorbed by PSII antenna, to the reduction of Q_B) and the total fluorescence basis (PI_{total}: the energy conservation from photons absorbed by PSII antenna, until the reduction of PSI acceptors) used for electron transport at 48 h increased by 7.27% (P<0.05), 33.99% (P<0.05) and 41.45% (P<0.05), respectively, compared with the control. The PI_{ABS} is a more sensitive parameter than F_v/F_m. Fructose significantly improved PI_{ABS} and PSI end acceptors (PI_{total}) at 48 h. The increase in PI_{ABS} indicated that the energy conservation ability and photosynthetic apparatus activity were improved (Hajihashemi *et al*., 2020). Our results indicated that the



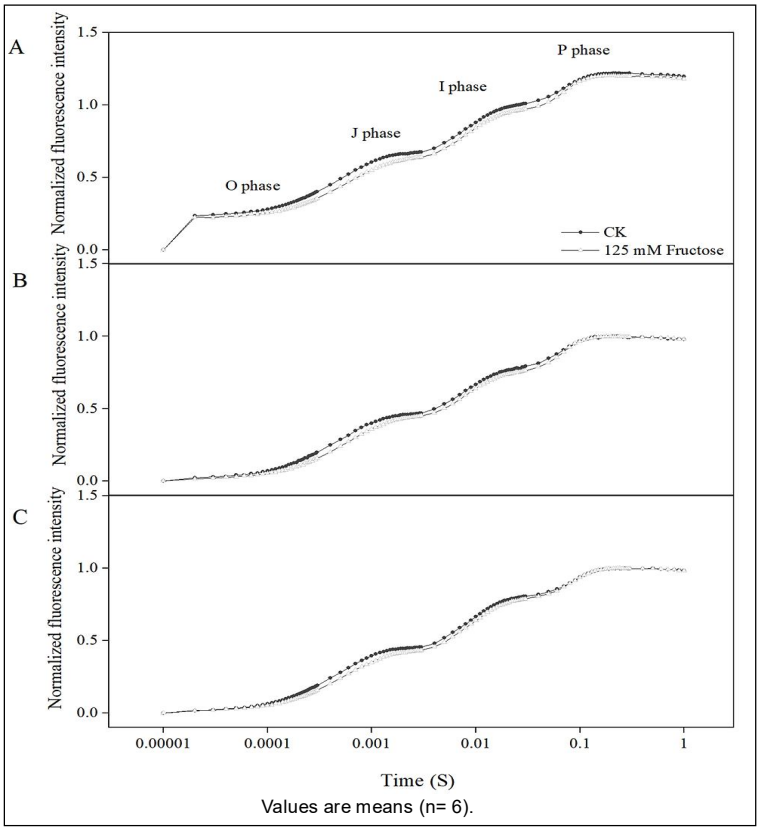
(A) Effects of CAT, DPI, SHAM and GSH on fructose-induced ROS production. (B) Dosage effect of fructose induced ROS production in guard cells of broad bean. Bars without a common letter are significantly different among treatments as determined by ANOVA (LSD test, P<0.05).

Fig 3: Fructose-triggered ROS production in guard cells of broad bean.



Pn (A), *Tr* (B), *Gs* (C), *Ci* (D), CE (E), LS (F), VPD (G), WUE (H) and stomatal aperture (I) of broad bean leaves at different time points after treatment with 125 mM fructose. Data represent mean±SE. Values of gas exchange parameters are demonstrated as means of three replicates along with standard deviation (SD; n= 4). The data of stomatal aperture in broad bean leaves are the mean of 120 measurements. ** and * indicate significant difference at the $P<0.01$ and $P<0.05$ between the treated and control plants, respectively. One-way ANOVA was performed and means differences were tested by LSD test.

Fig 4: The effects of fructose on the gas exchange and stomatal aperture of broad bean leaves over a time period of 48 h.



Values are means (n= 6).

Fig 5: Changes in the OJIP in *Vicia faba* leaves at 3 h (A), 24 h (B) and 48 h (C) under control (closed circle) and 125 mM fructose (open triangle) conditions and plotted on a logarithmic time scale.

Table 1: Changes in ABS/RC, DI_O/RC, TR_O/RC, ET_O/RC, RE_O/RC, PI_{total}, PI_{ABS}, φ_{PO}, Ψ_{EO}, φ_{EO} and δ_{RO}, φ_{RO} at 3 h, 24 h and 48 h after fructose and control treatments in broad bean.

Parameter	Treatment	Time (h)		
		3	24	48
ABS/RC	CK	1.66±0.085[a]	1.75±0.13[a]	1.72±0.079[a]
	Fructose	1.43±0.050[b]	1.43±0.052[a]	1.48±0.043[b]
DI_O/RC	CK	0.30±0.025[a]	0.33±0.036[a]	0.30±0.024[a]
	Fructose	0.25±0.010[a]	0.26±0.0094[a]	0.25±0.0078[a]
TR_O/RC	CK	1.35±0.060[a]	1.42±0.096[a]	1.42±0.056[a]
	Fructose	1.17±0.040[b]	1.18±0.043[b]	1.23±0.037[a]
ET_O/RC	CK	0.75±0.017[a]	0.77±0.032[a]	0.78±0.015[a]
	Fructose	0.69±0.018[b]	0.67±0.014[b]	0.72±0.015[b]
RE_O/RC	CK	0.28±0.0037[a]	0.29±0.0055[a]	0.27±0.0060[a]
	Fructose	0.27±0.0049[a]	0.28±0.00262[a]	0.26±0.0054[a]
PI_{ABS}	CK	3.59±0.46[a]	3.22±0.48[a]	3.56±0.42[a]
	Fructose	4.73±0.33[a]	4.38±0.38[a]	4.77±0.30[b]
PI_{total}	CK	2.19±0.33[a]	2.09±0.39[a]	1.93±0.25[a]
	Fructose	3.14±0.31[a]	3.19±0.34[a]	2.73±0.24[b]
φ_{PO}	CK	0.82±0.0055[a]	0.81±0.0060[a]	0.83±0.0056[a]
	Fructose	0.82±0.0015[a]	0.82±0.0085[a]	0.83±0.0022[a]
Ψ_{EO}	CK	0.56±0.012[a]	0.55±0.014[a]	0.55±0.011[a]
	Fructose	0.59±0.0080[a]	0.57±0.013[a]	0.59±0.0082[b]
φ_{EO}	CK	0.46±0.013[a]	0.44±0.014[a]	0.46±0.012[a]
	Fructose	0.48±0.0068[a]	0.47±0.011[a]	0.49±0.0073[a]
δ_{RO}	CK	0.38±0.0080[a]	0.38±0.15[a]	0.35±0.0066[a]
	Fructose	0.40±0.092[a]	0.42±0.0071[b]	0.36±0.0097[a]
φ_{RO}	CK	0.17±0.0078[a]	0.17±0.0011[a]	0.16±0.0062[a]
	Fructose	0.19±0.0061[a]	0.20±0.0062[a]	0.18±0.0057[a]

Data are the mean±SE (n= 6). Values without a common letter are significantly different as determined by ANOVA (LSD test, P<0.05).

trans-thylakoid sub-gradient was increased (Baghbani *et al*., 2019) and light chemical efficiency was high after fructose treatment. The increase in ψ_{EO} reflected the increase in the quantum yield of captured light energy used for electron transport downstream of Q_A.

CONCLUSION

In this study, fructose-induced stomatal closure was found to be dose- and time-dependent, with maximum effects being observed at 125 mM and 30 min. NO, Ca^{2+}, aquaporin and ROS were all involved in fructose-induced stomatal closure. Exogenous application of fructose can improve photochemical efficiency, *Pn* and WUE. This result serves as a reference for further investigation into the impact of sugar on plant physiology.

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Conflict of interest

All authors declared that there is no conflict of interest.

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