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In silico, in vitro and *in vivo* approach in understanding the functional relationship between ergosterol and Rubisco

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Abstract

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) is one of the key enzymes involved in assimilation of CO_2 in chloroplasts. Phylloplane microfungi and their metabolites have been reported to affect the physiology of host plants, particularly, their photosynthesis. However, information is lacking on the effect of these microflora on the physiology of chloroplasts. The current study emphasized the impact of two dominant phylloplane fungi, *Aspergillus niger* and *Fusarium oxysporum*, on activity of Rubisco in tomato chloroplasts. Ergosterol, which is a component of only fungal cell membranes and is not synthesized by plants, have been demonstrated to elicit activity of Rubisco. In the present study, it was demonstrated through *in silico, in vitro*, and *in vivo* approaches. Results demonstrated that the fungal metabolites, which contained ergosterol, could double Rubisco activity. Maximum carboxylation rate of Rubisco increased also in ergosterol-treated plants. Michaelis-Menten constant of Rubisco was also slightly affected. Ergosterol was found also to influence and enhance the binding of CO_2 and ribulose-1,5-bisphosphate to Rubisco. Therefore we can postulate that the physiology of the chloroplast is probably influenced by phylloplane microfungi.

Additional key words: enzyme activity; ergosterol; phylloplane; Rubisco; tomato.

Introduction

Chloroplasts harbour an important protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) which catalyzes the first step in photosynthetic assimilation of CO_2. Light modulation in plants is dependent on redox regulation of the larger isoform of Rubisco (Zhang *et al.* 2002). The carboxylase activity of Rubisco (EC 4.1.1.39) generates carbohydrate moieties essential for sugar synthesis through the Calvin cycle (Portis Jr. 1992, Meenakshi and Srisudha 2012).

Phylloplane microbes have been reported to support plant growth and development (Compant *et al.* 2005). Ergosterol, a key component of fungal cell membranes, is often found to be one of the common entities of the metabolite composition. Ergosterol is of fungal origin (Weete *et al.* 2010) and has not been reported till date to be produced by plants. Ergosterol has been reported to act as plant defense elicitors (Boller 1995, Felix *et al.* 1999). Kauss and Jeblick (1996) demonstrated that plant cells could perceive ergosterol which could elicit H_2O_2 production in the host plant. However, no data is available on the effect of ergosterol on chloroplast functioning. How these microbes or their metabolites influence plant physiology, particularly that of chloroplasts, is still an enigma. The present investigation could help understand the effect of phylloplane microfungal metabolite and ergosterol on the activity of Rubisco, which can be crucial in overall photosynthetic yield.

Materials and methods

Plant: *Solanum lycopersicum* plants were raised in plastic trays (25 cm × 24 cm × 6 cm) in sterile artificial soil under aseptic conditions at 25 ± 1°C, relative humidity of 70%, and 12 h (light/dark) photoperiod. Ten-week-old tomato plants were used for the study.

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Abbreviations: K_m – Michaelis-Menten constant; LSU – large subunit; OD – optical density; RuBP – ribulose-1,5-bisphosphate; SSU – small subunit; YME – yeast malt extract; V_{max} – maximum carboxylation rate.
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Fungi: *Aspergillus niger* and *Fusarium oxysporum* were isolated from leaf surfaces of tomato plants grown in Organic Farms, Amity University, NOIDA, India (Mitra *et al.* 2014).

Pathogen: *Pseudomonas syringae* pv. *tomato* was isolated from the infected fruits and maintained on King's B media (Aneja 2003).

Ergosterol: Ergosterol was procured from *Sigma*.

Extraction of fungal metabolites: *A. niger* and *F. oxysporum* (10^5 spores ml^{-1}) were inoculated separately in yeast malt extract (YME; Savoie and Mata 2003) broth and incubated at 25±1°C for 7 d on an orbital shaker at 70 rpm. The supernatant was membrane-filtered (0.22 μm) and used as metabolite samples.

Treatment of plants and sampling: Tomato plants were divided into eleven groups of 75 plants each. The treatment was as follows:

Group	Treatment
I	Sprayed with sterile distilled water only (control)
II	Inoculated with *P. syringae* pv. *tomato*
III	Treated with YME broth only
IV	Treated with *F. oxysporum* metabolite only
V	Treated with *A. niger* metabolite only
VI	Treated with *F. oxysporum* metabolite simultaneously inoculated with *P. syringae* pv. *tomato* inoculation
VII	Treated with *A. niger* metabolite simultaneously inoculated with *P. syringae* pv. *tomato* inoculation
VIII	Inoculated with *P. syringae* pv. *tomato* 24 h prior to treatment with *F. oxysporum* metabolite
IX	Inoculated with *P. syringae* pv. *tomato* 24 h prior to treatment with *A. niger* metabolite
X	Treated with *F. oxysporum* metabolite 24 h prior to inoculation with *P. syringae* pv. *tomato*
XI	Treated with *A. niger* metabolite 24 h prior to inoculation with *P. syringae* pv. *tomato*

Leaves of the third node from apex were tagged and subsequently sampled for all the groups at 0, 24, 48, 72, and 96 h. Three replicates were taken for each sample for each group.

Chloroplast isolation: Intact chloroplasts were isolated from the leaves of tomato as previously described by Diekmann *et al.* (2008) and Mitra *et al.* (2014). The leaf tissue was homogenized in grinding buffer containing 1.25 M NaCl, 50 mM Tris-HCl pH 8.0, and 7 mM EDTA. The chloroplast pellets were obtained at 4,000 rpm. Intact chloroplasts were obtained through sucrose gradient centrifugation and were washed four times with wash buffer to ascertain their intactness. The protein concentration was estimated according to Bradford (1976) and maintained at uniform amount (0.85 mg ml^{-1}) for all the tests.

Extraction of Rubisco and estimation of its activity: Rubisco was extracted from the isolated chloroplast pellets using Tricine buffer (Theobald *et al.* 1998, Bota *et al.* 2002, Mitra *et al.* 2014). Rubisco activity was estimated according to protocol of Wang *et al.* (2011). The reaction mixture consisted of 5 mM NADH solution, 50 mM ATP solution, Rubisco extraction solution, 50 mM creatine phosphate solution, 0.2 mM $NaHCO_3$ solution, Tris-HCl-EDTA-$MgCl_2$ reaction buffer (pH 7.8), 160 U ml^{-1} creatinephosphokinase, 160 U ml^{-1} phosphoglycerate kinase, 160 U ml^{-1} glyceraldehyde-3-phosphate dehydrogenase, and distilled water. The reaction mixture was incubated for one min and then the enzyme activity was determined from optical density (OD) at 340 nm (*UV-VIS, Shimadzu 1650*, Japan). The experimentally taken molar extinction coefficient for NADH was 2.66 mM^{-1} cm^{-1}. Enzyme activity was expressed as μmol g^{-1}(protein) min^{-1}. Maximum carboxylation rate (V_{max}) and Michaelis-Menten constant (K_m) value of the enzyme were also determined.

Ergosterol estimation in microfungal metabolites and washings of leaves treated with metabolites: To 3 ml of the metabolite or leaf washings, 3 ml of 25% alcoholic KOH solution and 60 μl of 10% pyrogallol solution was added. The solution was vortexed for 1 min. The mixture was then incubated at 85°C in a water bath for 1 h (Arthington-Skaggs *et al.* 1999, Tardieu *et al.* 2007). Ergosterol was extracted and estimated as described by Arthington-Skaggs *et al.* (1999).

***In silico* interactions between ergosterol and Rubisco**: The molecular interactions between Rubisco model and ligands were analyzed using *Hex Version 8.0.0*, *Schrodinger Maestro 9.7* and *LigPlot+* softwares (Kellenberger *et al.* 2004, Laskowski and Swindells 2011, Ghoorah *et al.* 2013). Multiple modulators which included CO_2, O_2, ribulose-1,5-bisphosphate (RuBP), 2-carboxy-arabinitol-1-phosphate (CA1P), and ergosterol units were docked to large (LSU) and small (SSU) subunits of Rubisco. Out of the above stated ligands, the binding properties of ergosterol to Rubisco LSU and SSU were extensively studied to understand the influence of the sterol in functioning of the protein.

***In vitro* interaction of ergosterol with extracted Rubisco**: The extracted Rubisco from control plants was tested for its activity in presence of purified ergosterol

(*Sigma*). Five different concentrations (0.5, 1, 3, 5, and 7 mg) of pure ergosterol per ml of ethanol was mixed with extracted Rubisco and incubated for 1 h. Subsequently, activity of Rubisco was estimated. Rubisco without ergosterol served as control.

***In vivo* interaction of ergosterol with Rubisco**: Aseptically raised plants were treated with the above mentioned concentrations of ergosterol. The leaves were sampled at 30-min intervals, and washed thoroughly five times with sterile distilled water to remove any traces of ergosterol. The washed leaves were then macerated to extract Rubisco and study its activity under the influence of ergosterol.

Statistical analysis of Rubisco activity and its kinetics was performed by *SAS* software (*version 9.2*) developed by *SAS Institute Inc*., Cary, NC, USA using two-way analysis of variance (*ANOVA*) and the least square means test followed by post hoc comparisons using *Tukey*'s honest significant differences.

Results

Ergosterol in fungal metabolites: The metabolite of *A. niger* contained 0.018 mg(ergosterol) ml^{-1}, whereas *F. oxysporum* had 0.013 mg(ergosterol) ml^{-1}. The leaf washings of the tomato plants sprayed with the fungal metabolites also contained traces of ergosterol (Table 1). Control, plants treated only with the bacterial pathogen and with YME media, did not have any traces of ergosterol, confirming its fungal origin.

Rubisco activity: Ten-week-old tomato plants inoculated with *P. syringae* pv. *tomato* showed a reduced Rubisco activity. YME media did not induce any changes in the Rubisco activity.

The Rubisco activity increased when the plants were treated with the metabolite of *A. niger* after 72 h. Application of *A. niger* metabolite simultaneously with the pathogen inoculation, *A. niger* metabolite applied 24 h prior to pathogen inoculation, and metabolite sprayed 24 h after pathogen inoculation resulted in significantly high Rubisco activity after 72 h (Fig. 1*A*).

Fusarium oxysporum metabolite, when applied simultaneously with pathogen, could enhance the Rubisco activity after 72 h, however, the activity increased after 96 h, when plants were treated with the metabolite prior to pathogen inoculation. However, the plants inoculated with *P. syringae* pv. *tomato* 24 h prior to metabolite application showed a reduced enzyme activity after 48 h as compared to other treatments. The overall impact of *F. oxysporum* metabolites was significantly much lesser than that of *A. niger* (Fig. 1*B*).

Kinetic properties: The V_{max} of Rubisco was significantly high in metabolite-treated plants. V_{max} and K_m value declined in pathogen-inoculated plants. *A. niger* metabolite was more effective than that of *F. oxysporum* in enhancing K_m value of Rubisco. The plants treated with metabolite of *A. niger* simultaneously along with the inoculated pathogen could induce maximum increase in V_{max} values, followed by plants treated with the fungal metabolite 24 h prior pathogen inoculation. Similarly, in tomato plants, the values were high. Similarly V_{max} value of Rubisco were high in tomato plants when treated with *F. oxysporum* metabolites either simultaneously or prior to pathogen inoculation. Plants treated with either of the metabolites after pathogen inoculation also had high V_{max}, though it was not as significant as in other treatments (Table 2). Thus, the results indicated that inhibitory effects of the pathogen on Rubisco activity could not be completely reversed by these metabolites. The K_m values of the protein were not significantly affected.

Table 1. Concentration of ergosterol in leaf washings. The indicated values were averaged for three replicates ± SD. Values followed by *different letters* within each column are significantly different from each other ($\alpha = 0.05$).

Treatment	Ergosterol [g ml^{-1}]
Control	0^a
Inoculation with *P. syringae* pv. *tomato*	0^a
YME media	0^a
F. oxysporum metabolite	0.22 ± 0.015^b
A. niger metabolite	0.421 ± 0.025^c
Co-inoculation with pathogen and *F. oxysporum* metabolite	0.247 ± 0.016^b
Co-inoculation with pathogen and *A. niger* metabolite	0.43 ± 0.017^c
Inoculation with pathogen 24 h prior to *F. oxysporum* metabolite spray	0.201 ± 0.021^b
Inoculation with pathogen 24 h prior to *A. niger* metabolite spray	0.46 ± 0.0225^c
F. oxysporum metabolite application 24 h prior to pathogen inoculation	0.273 ± 0.011^b
A. niger metabolite application 24 h prior to pathogen inoculation	0.402 ± 0.013^c

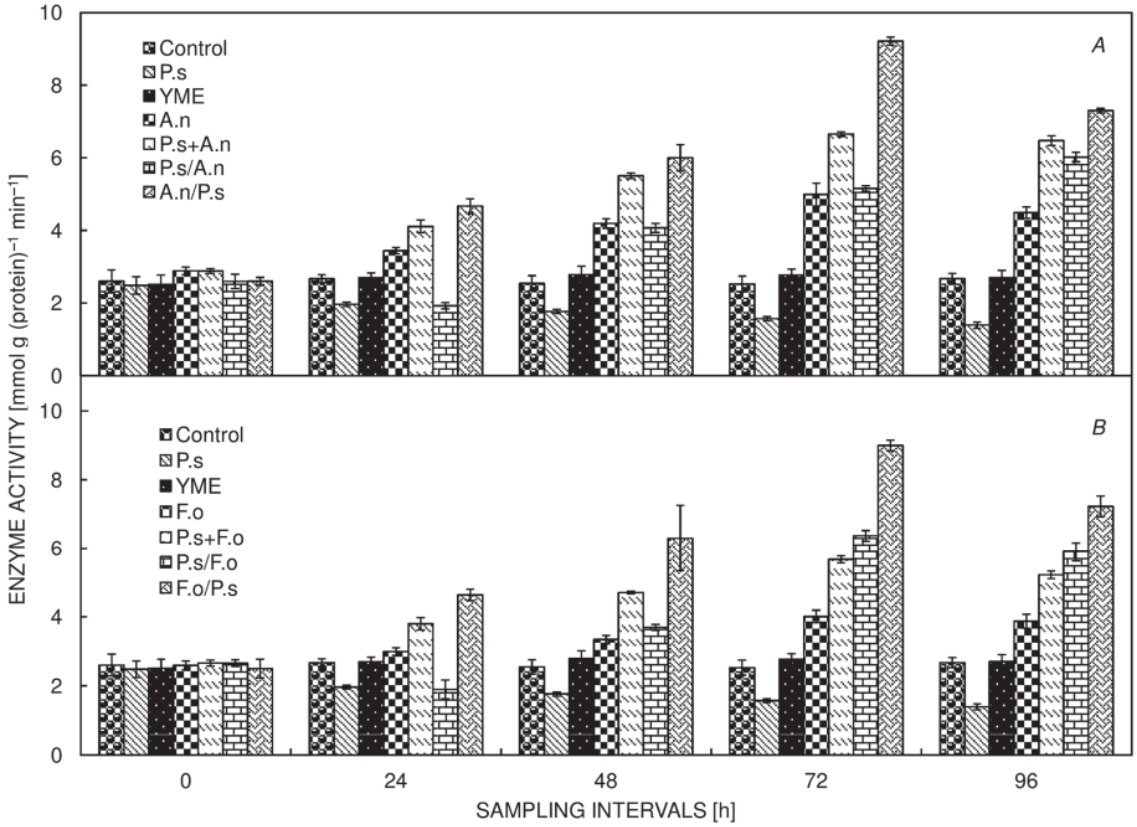

Fig. 1. Rubisco activity in ten-week-old tomato plants treated with (*A*) *Aspergillus niger* metabolite or (*B*) *Fusarium oxysporum* metabolite. P.s – inoculated with *Pseudomonas syringae* pv. *tomato*; YME – treated with YME media; A.n – treated with *A. niger* metabolite*;* P.s+A.n – co-inoculation of pathogen and *A. niger* metabolite; P.s/A.n – inoculated with the pathogen 24 h prior to *A. niger* metabolite spray; A.n/P.s – *A. niger* metabolite application 24 h prior to pathogen inoculation. F.o – treated with *F. oxysporum* metabolite*;* P.s+F.o – co-inoculation of pathogen and *F. oxysporum* metabolite; P.s/F.o – inoculated with the pathogen 24 h prior to *F. oxysporum* metabolite spray; F.o/P.s – *F. oxysporum* metabolite application 24 h prior to pathogen inoculation. Vertical bars represent SD.

Table 2. Maximum carboxylation rate (V_{max}) and Michaelis-Menten constant (K_m) of Rubisco in ten-week-old treated tomato plants. *Pseudomonas syringae* infected – inoculated with *P. syringae* pv. *tomato*; YME sprayed – treated with YME media; *A. niger* metabolite – treated with *A. niger* metabolite*; P. syringae + A. niger* – co-inoculation of pathogen and *A. niger* metabolite; *P. syringae/A. niger* – inoculated with the pathogen 24 h prior to *A. niger* metabolite spray; *A. niger/P. syringae – A. niger* metabolite application 24 h prior to pathogen inoculation; *Fusarium oxysporum* metabolite – treated with *F. oxysporum* metabolite*; P. syringae + F. oxysporum* – co-inoculation of pathogen and *F. oxysporum* metabolite; *P. syringae/F. oxysporum* – inoculated with the pathogen 24 h prior to *F. oxysporum* metabolite spray; *F. oxysporum /P. syringae – F. oxysporum* metabolite application 24 h prior to pathogen inoculation. The indicated V_{max} and K_m values were averaged for three replicates ± SD. Values followed by *different letters* within each column are significantly different from each other ($\alpha = 0.05$).

Treatment	V_{max} [mmol g(protein)$^{-1}$ min^{-1}]	K_m [mmol]
Control	12.73 ± 2.6^{b}	2.898 ± 0.0025^{b}
P. syringae infected	9.34 ± 1.9^{a}	2.071 ± 0.0014^{a}
YME sprayed	15.394 ± 3.21^{b}	3.0087 ± 0.00117^{b}
A. niger metabolite	19.534 ± 2.26^{c}	3.264 ± 0.0031^{b}
P. syringae + A. niger	28.36 ± 3.002^{d}	3.404 ± 0.002^{bc}
P. syringae/A. niger	20.83 ± 1.94^{c}	3.315 ± 0.00174^{b}
A. niger/P. syringae	26.257 ± 1.993^{d}	3.33 ± 0.00196^{bc}
F. oxysporum metabolite	16.895 ± 2.017^{b}	2.636 ± 0.0037^{b}
P. syringae + F. oxysporum	22.922 ± 2.146^{cd}	2.94 ± 0.0052^{b}
P. syringae/F. oxysporum	19.0083 ± 2.228^{c}	2.783 ± 0.00126^{b}
F. oxysporum/P. syringae	21.018 ± 2.93^{c}	2.993 ± 0.00224^{b}

Role of ergosterol in influencing Rubisco activity

***In silico* studies**: Among all the modulators, ergosterol was found to have a strong binding affinity to both the subunits of Rubisco. Ergosterol enhanced the affinity of CO_2 to the LSU of Rubisco. Binding of Rubisco to multiple ergosterol units increased the affinity of its large subunits to RuBP by 15–20 units (Fig. 2). The presence of the fungal sterol enhanced the Rubisco binding activity with its substrates. This suggests that ergosterol influences the chloroplast protein activity. Binding of ergosterol to Rubisco LSU also reduced the binding affinity of CA1P and O_2, the inhibitors of Rubisco. *In silico* studies showed that ergosterol plays a regulatory role in Rubisco activity.

***In vitro* and *in vivo* studies**: *In vitro* studies showed that the activity of Rubisco extracted from the tomato plants was significantly enhanced with an increase in concentration of ergosterol till 3 mg ml^{-1} (Fig. 3). Similarly, *in vivo* studies demonstrated that 3 mg(ergosterol) ml^{-1} was the most significant in eliciting Rubisco activity as compared to 1 mg ml^{-1}. However, higher concentrations did not elicit any further increase in activity. The metabolites of *A. niger* and *F. oxysporum* containing ergosterol could also elicit enzyme activity (Fig. 3).

Discussion

Pathogens cause retardation of Rubisco catalytic activity along with reduction of K_m value. Bowes (1991) reported pathogen inoculation could reduce a content of Rubisco in plants. Microfungal metabolites may inhibit the retardation of Rubisco activity induced by pathogen attack. The present study also demonstrated that metabolite of two phylloplane microfungi, *A. niger* and *F. oxysporum*, regulated positively the Rubisco activity, which might influence the overall photosynthesis.

Dixon *et al.* (1994) identified ergosterol as fungal compounds displaying elicitor activities in various plant systems. Ergosterol was found to be effective in eliciting synthesis of reactive oxygen species in tobacco plants (Kašparovský *et al.* 2004, Vatsa *et al.* 2011). Plants have been reported to potentially recognize ergosterol through recognition receptors and to initiate an efficient defense response (Kloppholz *et al.* 2011). The plant cell wall has an average pore size of 35–70 Å (Carpita *et al.* 1979) and the plasma membrane bears 5–20 Å pores (Pérez-Donoso *et al.* 2010). No information is available regarding of ergosterol entering into plant cells, but it might influence the activity of Rubisco through interaction with

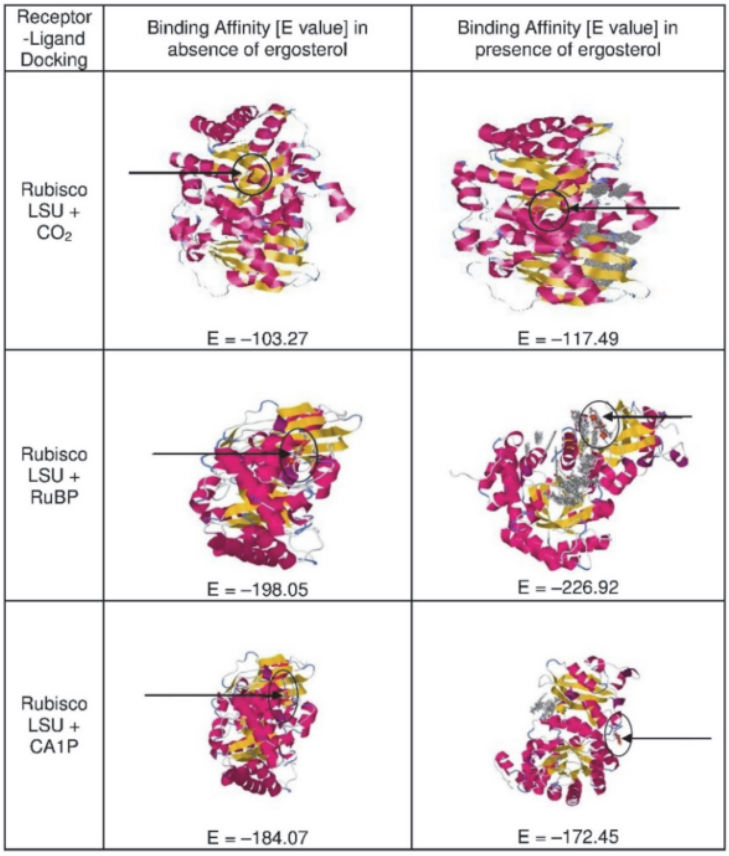

Fig. 2. Docking scores in large subunit of Rubisco in tomato. Rubisco LSU + CO_2 – CO_2 docked to the large subunit of Rubisco; Rubisco LSU + RuBP – RuBP docked to the large subunit of Rubisco; Rubisco LSU + CA1P – CA1P docked to the large subunit of Rubisco.

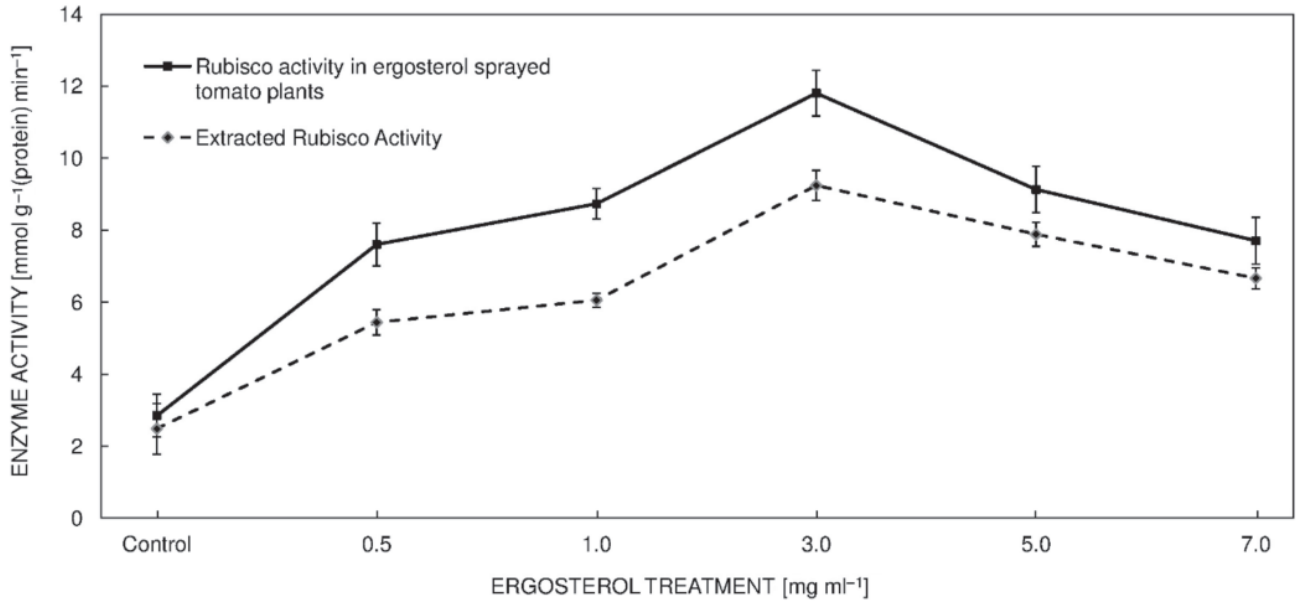

Fig. 3. *In vitro* effect of ergosterol on Rubisco activity. Vertical bars represent SD.

plant receptors. Ergosterol was found to induce changes in proton fluxes and membrane hyperpolarization in plant motor cells (Amborabé *et al.* 2003). It is distinctly perceived by plant cells and triggers defense responses at the plasma membrane level (Dadáková *et al.* 2013). The current observation that phylloplane microfungi secrete ergosterol, which was detected in phylloplane washings, and ergosterol influences Rubisco activity may indicate that the ergosterol from the phylloplane microfungi might be critical in regulation of chloroplasts. The plants source their ergosterol from phylloplane microfungi who have the exclusive mechanism to synthesise it.

In silico studies demonstrated the binding of ergosterol to Rubisco which affected the binding of RuBP to the protein. The fungal sterol, when bound to Rubisco, increased the binding affinity of RuBP as well as CO_2 to the enzyme (Fig. 2). It is well known that Rubisco activity is highly dependent on the activity of Rubisco activase. Rubisco activase activity is modulated by the chloroplast redox status and ADP/ATP ratios, thereby mediating Rubisco activation and photosynthetic induction in response to irradiance (Carmo-Silva *et al.* 2014). Rubisco activase facilitates the removal of CA1P from carbamylated sites of Rubisco (Parry *et al.* 2003). CA1P is a known natural inhibitor of Rubisco, and its removal from the active site of the enzyme is very much needed for the carbon fixation cycle to proceed without any hindrance. *In silico* docking of ergosterol to Rubisco also reduced the binding affinity of CA1P, thus possibly mimicking the role of Rubisco activase (Fig. 2). Similarly, ergosterol was also found to reduce O_2-binding affinity to Rubisco. The oxygenation reaction competes with carboxylation and reduces photosynthetic productivity (van Lun *et al.* 2014). *In silico* experiments with ergosterol highlighted a hypothetical action mechanism of ergosterol in influencing the functioning and activity of Rubisco. Though clear data are not available on ergosterol entering the plant cytoplasm, the present data clearly demonstrated that fungal sterols were involved in fixation of CO_2 by Rubisco.

In vitro and *in vivo* studies supported the findings that ergosterol induce Rubisco activity in tomato. The current study focused on the role of ergosterol in Rubisco activity. The sterol was effective in regulating the enzyme activity and could also suppress the pathogenic effects of *P. syringae*.

Our results thus demonstrated the potential of phylloplane microfungal metabolites containing ergosterol in influencing Rubisco activity in chloroplasts. The present study attempted to prove the existence of a positive correlation between ergosterol and Rubisco activity. A functional correlation between phylloplane microfungi and Rubisco activity highlighted the possibility of coevolution of higher plant chloroplasts and leaf surface microfungi. Ergosterol from these fungi provided a regulation of Rubisco activity for optimal photosynthetic yield.

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