Impact of the facultative parasitic weed *Rhamphicarpa fistulosa* (Hochst.) Benth. on photosynthesis of its host *Oryza sativa* L.

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ABSTRACT

Rhamphicarpa fistulosa is a facultative root parasitic annual forb, of the family Orobanchaceae that is native to sub-Saharan Africa. Parasitism results in yield reductions by the host plants but it is not known how exactly *R. fistulosa* affects its host or how the host responds physiologically. In three pot experiments, we investigated whether and when the parasite affects photosynthesis of rice, whether the level of impact was parasite density dependent and explored mechanisms underlying the response of rice photosynthesis to parasitism. Photosynthesis and related parameters were measured at a range of light use intensities. Host photosynthesis was negatively affected while light use efficiency was negatively affected only later on in the growth process. Except for dark respiration rates, which were never affected by parasite infection, suppression of host photosynthesis at light saturation, the initial light-use efficiency, chlorophyll content, specific leaf area and shoot weight were parasite density dependent with a stronger effect for higher parasite densities. Already at 56 days after sowing, the slope of the linear relationship between light adapted quantum efficiency of PSII electron transport (Φ_{PSII}) and the quantum yield of CO_2 assimilation (Φ_{CO2}) of infected plants was less than those of un-infected plants. There was a considerable time lag between the parasite's acquisition of benefits from the association, in terms of growth (previously observed around 42 DAS), and the reduction of host photosynthesis (around 56 DAS). Expression of relative reductions in host growth rates started at the same time as the relative suppression of host photosynthesis. This indicated that *R. fistulosa* affects host growth by first extracting assimilates and making considerable gains in growth, before impacting host photosynthesis.

Keywords: Rice Vampireweed, rice, parasitic plants, CO_2 assimilation, stomatal conductance, quantum yield.

1 Introduction

In recent years, rice has become important as a food and cash crop in sub-Saharan Africa (SSA). As a result, the area under rice cultivation has expanded to an estimated 10 million hectares (Seck et al., 2012). Most of the expansion has been into marginal rainfed rice growing areas (Balasubramanian et al., 2007; Sakurai, 2006). Apart from suboptimal provision of water and nutrients for crops, these areas are natural habitats of parasitic weed species. It is estimated that in Africa, 1.34 million ha of rainfed rice is infested with at least one species of a parasitic weed causing economic losses of US $200 million (Rodenburg et al., 2016b). The economically most important genus *Striga* (Mohamed et al., 2006) is a well-known biotic production constraint to traditional staple food crops like sorghum, millet, maize and upland rice. Recently, the facultative parasitic weed *Rhamphicarpa fistulosa* has developed into an important pest of rainfed lowland rice (Rodenburg et al., 2015).

Rhamphicarpa fistulosa is a root hemi-parasitic annual forb species of the family Orobanchaceae that is native to SSA (Kuijt, 1969; Ouédraogo et al., 1999; Rodenburg et al., 2015). Its habitat range includes open sunny grasslands such as temporary wetlands or waterlogged soils (Hansen, 1975). As a root hemi-parasite, *R. fistulosa* possesses a haustorium, a specialized organ that forms the connection with the host enabling the host-parasite transfer of water, nutrients and metabolites (Kabiri et al., 2017; Kabiri et al., 2015; Kuijt, 1969). The species is facultative, meaning that it can complete its life cycle as a free-living plant (Kabiri et al., 2017; Ouédraogo et al., 1999). However, when in association with a host, the parasite grows taller, produces more biomass and has a higher fecundity (Kabiri et al., 2017; Kabiri et al., 2016). The parasitism has a negative effect on the rice host plant, resulting in severe yield reductions (Kabiri et al., 2017) ranging from 24-73% (Rodenburg et al., 2016a). Parasite effects on hosts range from negligible to severe, depending on the parasite and host species involved. For instance, while rice is severely affected by *R. fistulosa* infection, Seel and Press (1996) reported that infection of the perennial grass host *Poa alpina* by the facultative parasite *Rhinanthus minor* merely affected flower bud formation and biomass partitioning.

Apart from resource extraction, parasitic plants also impact their hosts by manipulating host-plant architecture like in the association between the hemi-parasite *Striga hermonthica* and its host sorghum. Van Ast et al., (2000) observed that internode lengths of a *Striga*-susceptible sorghum cultivar were two folds shorter in infested compared with un-infested plants. This reduced internode length causes a more compact canopy that leads to self-shading. In some host-parasite associations, the negative impacts parasitic plants have on their hosts is restricted to withdrawal of assimilates. For example, in an association of *Rhinanthus minor* and barley, the parasite trafficked up to 20% of host solutes from the xylem sap, reducing host growth by 22% (Jiang et al., 2003). Assimilate production of host plants has been reported to be inhibited through a direct negative impact of the parasite on the photosynthetic capacity of its host (Cameron et al., 2005; Watling and Press, 2001). These parasite-induced effects on host photosynthesis are both host and parasite specific (Watling & Press, 2001, Hibberd et al., 1998a). For example, with *Striga* spp. the largest effect on host yield was caused by the effect of the parasite on host photosynthesis (Cechin and Press, 1994; Gurney et al., 1995). Graves *et al.*, (1989) observed that in an association of *Striga hermonthica* and sorghum, 80% of the host yield loss was caused by parasite-induced suppression of host photosynthesis.

The degree of impact on host photosynthesis can be influenced by time and level of infection. For example, van Ast (2006) observed that, at high *S. hermonthica* infestation levels, a 40-50% reduction in photosynthetic rate of sorghum leaves was already observed at 19 days after sorghum emergence (DAE), well before the parasite emerged. A reduction in leaf photosynthetic rate was also observed at lower *Striga*-infestation levels, albeit to a lower extent. However, from 57 DAE and onwards, the reduction in leaf photosynthetic rate became independent of the level of *Striga* infestation. For *Rhamphicarpa fistulosa*, it is not known

whether the parasite impacts host plant photosynthesis and by which mechanism, an investigation of which comprises a research question that has both scientific value (because it concerns a facultative parasite) and economic value (because the parasite's distribution and importance as a weed to rice is on the increase). The objective of this study was to find out (i) if and at what time *R. fistulosa* affects photosynthesis of rice, (ii) if the level of reduction in leaf photosynthetic rate depends on parasite infection level and (iii) which mechanisms underlie a possible reduction in leaf photosynthetic rate of its rice host.

2 Materials and Methods

Between 2012 and 2014, three greenhouse pot experiments were conducted at Wageningen University, the Netherlands. Rice (cultivar IR64) was used as the host plant species. Seeds of *R. fistulosa* were from a seed lot collected in 2009 from an infested rice field in Kyela, Tanzania. The germination percentage of these seeds was between 48% and 60%. The pots used were unperforated 6 L capacity pots filled with approximately 7.1 kg of a 1:1 mixture of dry arable soil and sand. The pots were watered daily to maintain saturated soil conditions since wet soils are most suitable for germination, growth and development of *R. fistulosa*. Screens were used to create a day length of 12 h (from 7.00 a.m. to 7.00 p.m.). Growing conditions were set to 26˚C/23˚C for day/night temperatures, but during warm summer days daytime temperature was regularly higher with a maximum of 32ºC. Relative humidity varied between 50 and 70%. Supplementary lighting was provided by lamps (SON-T Agro, 400 W, Phillips) that automatically switched on when photosynthetically active radiation outside the greenhouse dropped below 910 $\mu Em^{-2}s^{-1}$.

In all experiments, rice seeds were pre-germinated for 48 hours in an incubator at 33˚C after which they were planted in the centre of a pot at a depth of 1-2 cm. Only one rice seedling was planted per pot. On the same day, *R. fistulosa* seeds were mixed with about 9 g of dry sand and evenly sprinkled on top of the soil surface, to facilitate daylight-dependent germination of the seeds. The surface was then moistened with a fine spray of water. After emergence, *R. fistulosa* seedlings were uniformly thinned to a pre-set density.

1.1 Experiment 1

Experiment 1 was carried out from May 16 to July 23, 2012, to investigate if *R. fistulosa* affected leaf photosynthetic rate of rice. The experiment consisted of a randomized complete block design with 4 uninfected and 4 infected plants for each gas exchange measurement recorded on six occasions. The treatments included rice plants infested with 30 *R. fistulosa* plants and un-infested plants. Leaf photosynthetic rate was determined at 30/31, 36/38, 44/45, 51/52, 58/59 and 65/66 days after sowing (presented as 30, 37, 44, 51, 58 and 65 DAS, respectively). The total number of pots was 48. Fertilizers were not applied. Relative chlorophyll content (SPAD) and specific leaf area (SLA), of host leaves used for gas exchange measurements, was also determined.

1.2 Experiment 2

Experiment 2 investigated the relation between the suppression in leaf photosynthetic rate and *R. fistulosa* infestation level. The experiment was conducted from May 5 to July 31, 2012. It was a randomized complete block design with one treatment (parasite infestation) comprising six treatment levels in four replicates. The treatment levels were rice plants infested with 7, 14, 28, 56 and 112 seeds of *R. fistulosa* per pot and an un-infested plant. Gas exchange measurements were conducted at 65 DAS as it had been noted in Experiment 1 that photosynthesis of the host was clearly influenced by parasite infection at this time. Gas exchange measurements on rice plants were conducted with the same irradiance levels in the same procedure as the first experiment. SPAD and SLA, of leaves on which gas exchange

measurements were done, were also measured. Aboveground biomass of both rice and *R. fistulosa* plants were harvested and the plants material dried at 70˚C for 48 hours and weighed to obtain shoot dry weight per plant.

1.3 Experiment 3

To investigate the mechanisms underlying the reduction in leaf photosynthetic rate of the rice host, a third experiment was installed from April 22 to July 9, 2014. The experiment had a randomized complete block design, with 3 infection levels (0, 13 and 6 parasites per pot) in 4 replicates, represented in 8 gas exchange measurements therefore giving a total number of 96. The gas exchange measurements were conducted at 29/30, 34, 40/41, 48/49, 55/56/57, 62/63/64, 69/70/71 and at 76/77 DAS (presented as 30, 34, 40, 48, 56, 63, 70 and 76 DAS). Irradiance levels were measured in descending order. At 34 DAS, gas exchange measurements were limited to the three highest light levels (2000, 1500, 1000 µE m^{-2} s^{-1}), due to limited availability of the photosynthesis equipment. At 56, 63 and 70 DAS gas exchange measurements were expanded with fluorescence measurements.

1.4 Gas exchange measurements

From around 30 DAS on, leaf photosynthetic rates of infested and un-infested rice plants were regularly determined. Gas exchange measurements were done on the middle of the adaxial side of the youngest fully developed leaf of the main tiller. The measurements were conducted during the day between 9.30 a.m. and 4.00 p.m. using photosynthesis measurement equipment (Li-6400XT, LI-COR, and Lincoln, NE, USA). Leaf temperature and the average relative humidity in the leaf chamber were maintained at 29.9°C and 66% respectively. For the first two experiments, leaves were first dark adapted for 15 min, after which light intensity was increased subsequently at irradiance levels (PAR) 0, 20, 50, 100, 500, 1000 and 1500 µE $m^{-2}s^{-1}$. At each irradiance level, the leaf was exposed for 6 minutes before a measurement was recorded. For the third experiment, additional irradiance levels were introduced, and the measurements were taken in descending order (2000, 1500, 1000, 500, 160, 120, 80, 40, 0 µE m^{-2} s^{-1}) to improve the light adaptation time of the leaf. The response of photosynthesis to irradiance was examined by fitting the exponential model of von Caemmerer and Farquhar (Caemmerer and Farquhar, 1981) for gas exchange in leaves to the measured data.

$$A = A_{max} \times \left(1 - exp\left(-\varepsilon \times \frac{PAR}{A_{max}}\right)\right) - R_d \quad (1)$$

In this model, A is the net assimilation rate (µmol CO_2 m^{-2} s^{-1}), A_{max} is the maximum gross assimilation rate (µmol CO_2 m^{-2} s^{-1}), ε is initial light use efficiency (µmol CO_2 μE^{-1}), R_d is the dark respiration rate (µmol CO_2 m^{-2} s^{-1}) and *PAR* is the photosynthetic active radiation (µE m^{-2} s^{-1}).

After the gas exchange measurements, leaf greenness and specific leaf area (SLA, i.e. the fresh leaf area divided by its dry weight, in m^2 kg^{-1}) of the measured leaf were determined. Leaf greenness was measured using a SPAD Chlorophyll Meter (SPAD 502, Spectrum Technologies, Inc., Plainfield, IL, USA), after which a 12-cm-long piece of the leaf was cut, and the leaf width was determined to then calculate the area. The leaf piece was then placed in an oven for 48 hours at 70˚C after which its dry weight was determined to derive SLA.

1.5 Fluorescence

While all of the above measurements were done in all three experiments, in the third and last experiment, additional observations were made on leaves of 56-, 63- and 70-day old rice plants. These measurements were conducted to determine whether kinetics of fluorescence induction

was altered in leaves of *R. fistulosa*-infested plants. The protocol followed for fluorescence measurements were done according to methods of Maxwell and Johnson (2000) using the Li-6400XT (LI-COR, Lincoln, NE, USA) equipment. Leaf temperature and the average relative humidity in the leaf chamber were maintained as above.

Dark adapted leaves were exposed continuously to the respective light irradiances outlined above producing a transient closure of the PS II photochemical reaction centres in a light adapted state (F'_m) until the fluorescence steady-state where fluorescence intensity had saturated or was constant (F_t). The fraction of absorbed photons used for photochemistry for a light adapted leaf known as the quantum yield of PS II ($\Phi PSII$) was calculated using Equation 2:

$$\Phi PSII = (F'_m - F_t)/F'_m \quad (2)$$

Quantum yield of CO_2 assimilation was calculated as:

$$\Phi CO_2 = (A - R_d)/\, PAR \quad (3)$$

Where, ΦCO_2 is the quantum yield of CO_2 assimilation based on incident radiation, A is the photosynthetic rate (μmol CO_2 m^{-2} s^{-1}), R_d is the dark respiration rate (μmol CO_2 m^{-2} s^{-1}), *PAR* is the incident photosynthetic active radiation (μE m^{-2} s^{-1}).

1.6 Statistical analysis

Curve fitting to obtain the photosynthetic parameters of the light response curve (Eq. 1) was performed using R version 2.1.5. The parameters estimated from the light response curves, SPAD values, specific leaf area and dry weight data were subjected to Analysis of Variance (ANOVA) using statistical software package Genstat for Windows 17th Edition (Genstat, 2016). ANOVA was preceded by tests for homoscedasticity and normality, as recommended by (Sokal and Rohlf, 1995), and followed by comparison of means using the Least Significant Difference test (LSD).

3 Results

Experiment 1

The relation between measured photosynthesis and photoactive radiation in Experiment 1, was well described by the exponential model of (Caemmerer and Farquhar, 1981) at all measurement dates (Fig. 1; R^2 value range: 0.941 to 0.999). The photosynthesis increased quickly at irradiances ranging from 0 to 100 irradiance levels, and plateaued in the range of 500 to 1,500 irradiance levels (Fig. 1). Photosynthesis-light response curves of infested and un-infested rice plants were the same until 58 days after sowing (DAS). The maximum photosynthetic rate (A_{max}) was attained at 1500 PAR. At 58 and 65 DAS the A_{max} (μmol CO_2 m^{-2} s^{-1}), of plants in infested pots was 43% (7.56; $P<0.021$) and 66% (5.88; $P<0.008$) less than that of un-infested plants (58 DAS; 13.27; 65 DAS; 17.20), respectively. In addition, the impact of the parasite on host photosynthesis was further observed in the maximum photosynthetic rate per gram of leaf dry weight (A_{mass}), of infested plants. The parasite reduced the A_{mass} of infested rice plants by 17% (i.e., 0.719; $P<0.022$) at 37 DAS and 42% (i.e., 0.221; $P<0.022$) at the end of the experiment, at 65 DAS (Table S1). The means of the A_{mass} of uninfected plants was 0.869 and 0.381 at 37 DAS and 65 DAS respectively.

The initial slope of the photosynthesis light response curves of un-infested plants, representing the initial light use efficiency (ε), was stable over time, with an average value of 0.069 (μmol CO_2 μE^{-1}) across measurement dates (data not shown). The initial light use efficiency (ε) was only significantly affected by *R. fistulosa* at 65 DAS (P=0.006; Table S1)

where ε of rice plants with *R. fistulosa* was three times less (0.021 µmol CO_2 μE^{-1}) than that of un-infested plants (0.060 µmol CO_2 μE^{-1}). Dark respiration rates, R_d, of infested rice plants decreased from 1.37 µmol CO_2 m^{-2} s^{-1} at 30 DAS to 0.49 µmol CO_2 m^{-2} s^{-1} at 65 DAS but did not differ from un-infested plants (*P* values: 0.198 - 0.703; Table S1) at any observation date. The relative chlorophyll content (SPAD values) of un-infested plants were stable over time with an average value of 35 while those of infested plants decreased after 51 DAS (Fig. 2A; Table S1). The SPAD values of rice plants with and without *R. fistulosa* only differed at 65 DAS (*P*=0.006), where values of plants growing with the parasite were half those of un-infested plants. An increase in specific leaf area (SLA) denoting thinner leaves of infested rice plants, was also observed. The SLA of un-infested plants gradually decreased from 42 m^2 kg^{-1} at 30 DAS to 22 at 65 DAS, while those of parasite infested plants decreased alongside over the first four measurement dates, but then increased again after 51 DAS (Fig. 2B; Table S1). The SLA of *R. fistulosa*-infested plants was 1.5 higher than un-infested plants at 58 DAS (*P*=0.006) and 1.7 times higher at 65 DAS (*P*=0.004; Fig. 2B; Table S1).

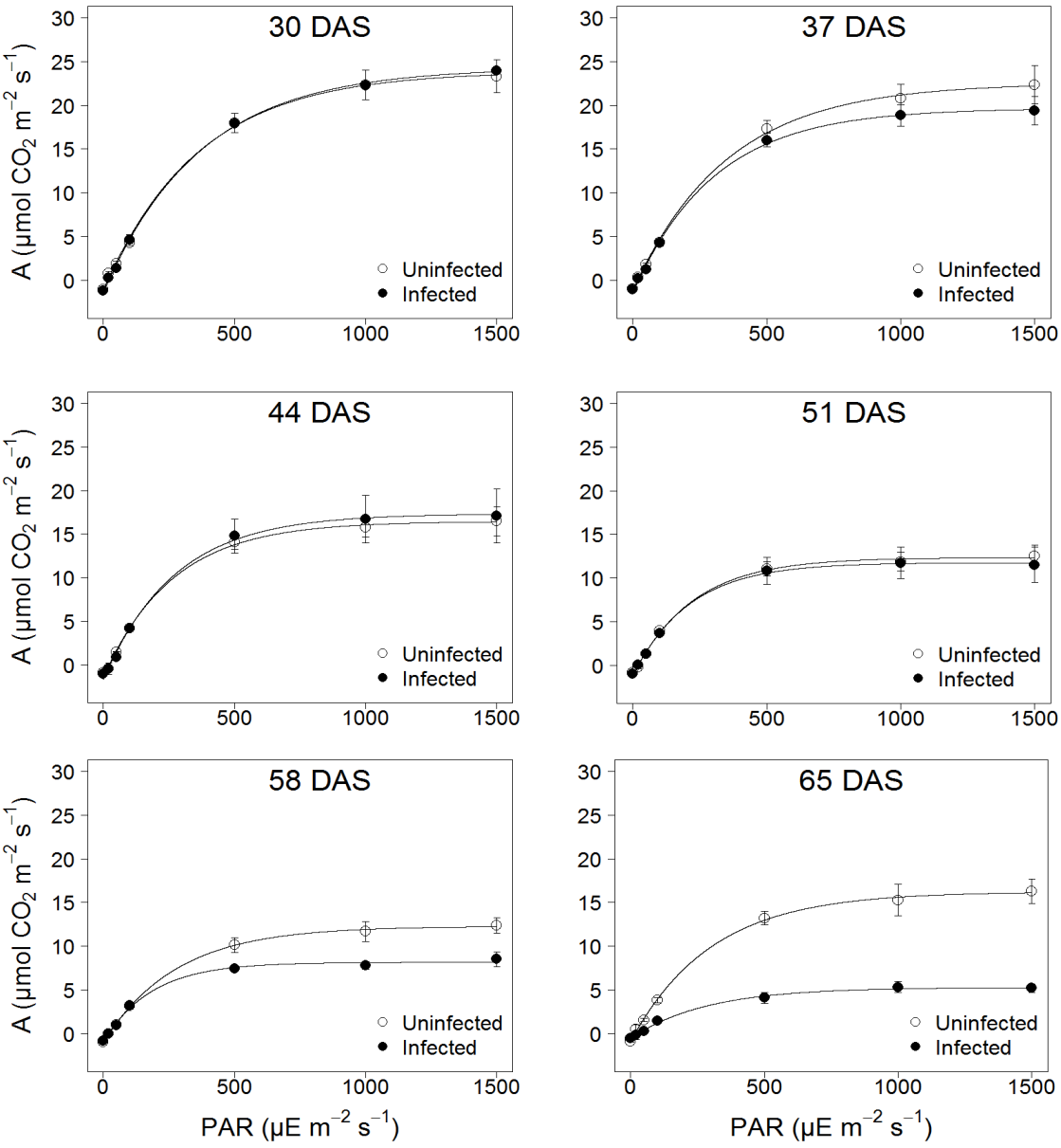

Figure 1. Time course of photosynthesis-light response curves for leaves from rice plants infested by *R. fistulosa* compared with leaves from un-infested rice plants (Experiment 1). Error bars are standard errors of mean. *N*=3 for infested plants at 58 DAS, otherwise *N*=4.

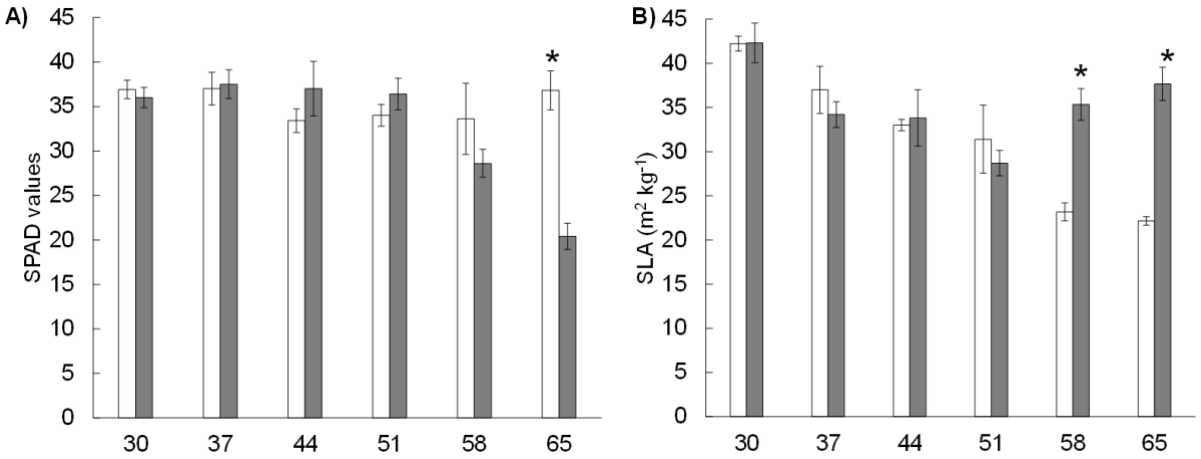

Figure 2. SPAD values (A), specific leaf area, SLA (B) of the youngest fully developed leaf of un-infested and *R. fistulosa* infested rice plants at different observation times (30, 37, 44, 51, 58 and 65 DAS; Experiment 1). Error bars are standard errors of mean. * indicates significant differences *(P*< 0.05*)* between infested and un-infested rice plants.

Experiment 2

Infestation significantly decreased host A_{max} at all parasite densities and most severely at 14, 28 and 112 parasite seeds per pot (Fig. 3A; Table S2). The impact of parasite density on the initial light use efficiency (ε) of the host was significantly (*P*=0.002) reduced by *R. fistulosa* at infestation rates of 14, 28 and 112 seeds per pot, but not at 7 and 56 seeds per pot (Fig. 3B; Table S2). Again, dark respiration rates (R_d) did however not differ between infested and un-infested plants irrespective of parasite infestation level (*P*<0.641; Table S2). The SPAD values were affected by *R. fistulosa* at infestation levels of 14 seeds per pot and higher (*P*<0.00), with the exception of the fourth infestation level (56 seeds per pot; Fig. 3C, Table S2). The SLA was affected by *R. fistulosa* at infestation levels of 14 seeds per pot and higher (*P*<0.001; Fig 3D, Table S2) but to variable degrees.

The host shoot dry weights differed from that of un-infested plants (*P*<0.001) at all infestation levels (Table S2), with the lowest shoot dry weight observed at the highest infestation level (Fig. 3E). The total aboveground *R. fistulosa* shoot dry weight per pot was proportional to the infestation levels (*P*<0.001; Fig. 3F). As a result from the above presented physiological effects from parasitism, the shoot dry weight of *R. fistulosa* infested rice plants showed a reverse proportional trend (Fig. 3F, Table S2).

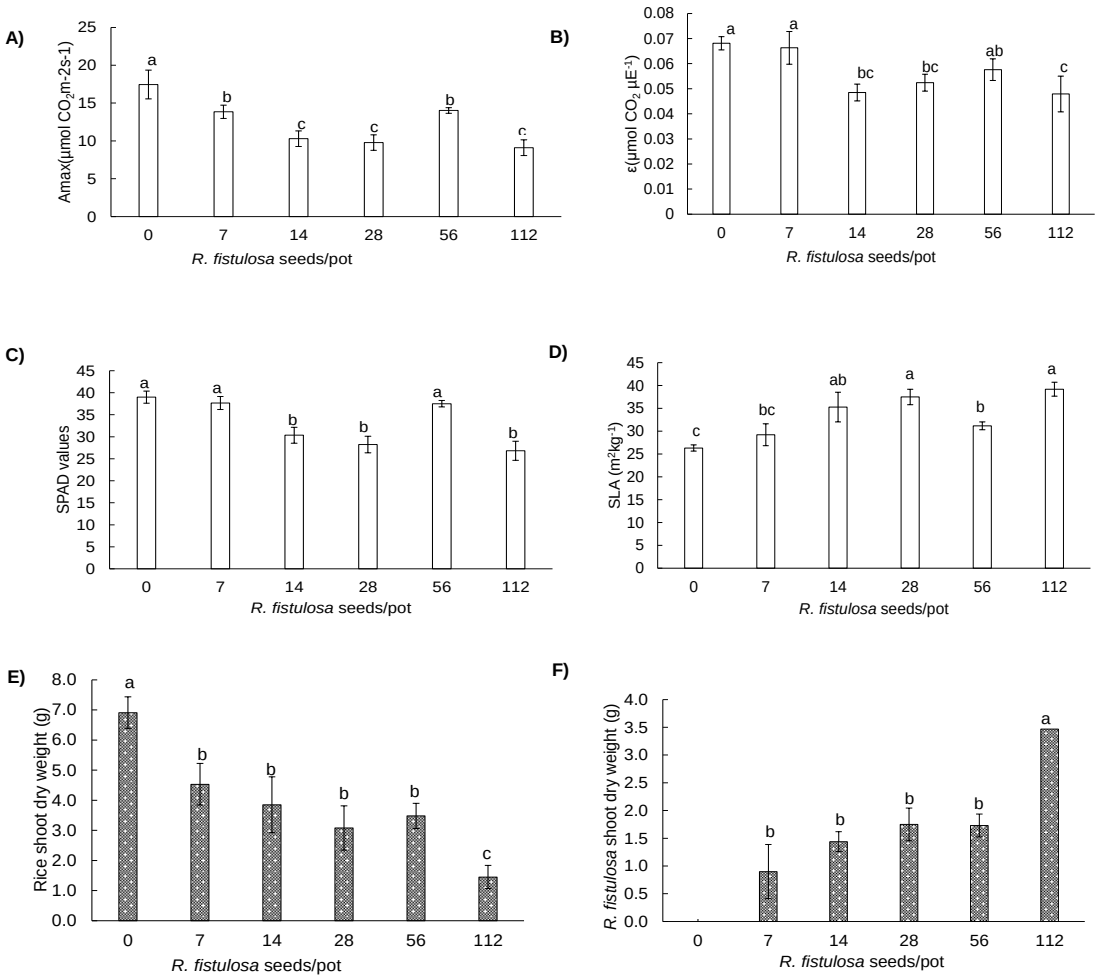

Figure 3. Maximum gross photosynthetic rate per unit leaf area, A_{max} (A), the initial light use efficiency, ε (B), SPAD values (C) and specific leaf area, SLA (D) of rice plants at 65 DAS as influenced by infestation levels of *R. fistulosa* (Experiment 2). (E) shows the above-ground dry weight of the host, and (F) is above-ground dry weight (per plant) of the parasite. Error bars are standard errors of mean. Bars with different letters were significantly different (P<0.05).

Experiment 3

In Experiment 3, irrespective of the parasite infestation level, the photosynthesis-light response curves of *R. fistulosa*-infested rice plants were similar to those of un-infested plants (Fig. 4 A-D) until 56 DAS; from 56 DAS onwards the light response curves were significantly reduced by infection (P<0.001; Fig. 4 E-H). Interaction effects of time (in DAS) x parasite density were found for the maximum photosynthetic rate (A_{max}; Table 1). Whereas the A_{max} of un-infested plants remained relatively stable beyond 49 DAS, the Amax levels of infested plants steeply decreased in time from that point onwards, with the steepest reduction observed at the highest parasite density (Table 1). In addition, interaction effects of time x parasite density were found for the maximum photosynthetic rate per gram of leaf dry weight (A_{mass}; Table 1). Parasite infestation significantly reduced A_{mass} of infested plants at observation times beyond 49 DAS in a similar way as A_{max}. Whereas the A_{mass} of un-infested plants also gradually decreased over time, the A_{mass} infested plant decreased more steeply, with the largest reductions observed at the highest infestation level (Table 1). The initial light use efficiency (ε) changed significantly

over time, with an initial increase at 41 DAS, compared to the fist observation date of 29 DAS, and a decrease beyond that point followed by an increase again at the latest date of 77 DAS. The ε was also negatively affected by parasite density, with a significant reduction at the highest density level compared to the un-infested plants (P=0.009; Table 1). Interaction effects of time x parasite density were found for SPAD values (P=0.005; Table 1). From 56 DAS onwards, parasite infestation significantly reduced SPAD but only significantly so at the highest infestation density level (i.e. 13 parasite plants). At the lower parasite density (i.e. 6 parasite plant), SPAD of host plants were not significantly reduced compared to un-infested plants. The difference in SPAD levels of host plants between the two parasite infestation levels was significant from 63 DAS onwards. There was also a significant interaction effect of time x parasite density observed for SLA values (P<0.001; Table 1). At the high parasite density level (i.e. 13 plants) the SLA of host plants was significantly higher than un-infested plants at 41, 63 and 70 DAS, whereas at the low parasite density level (i.e. 6 plants) the SLA of host plants was only significantly higher from that of un-infested plants at 63 DAS. More analysis of variance parameters of Experiment 3, is shown in Table S3 of the supplementary section.

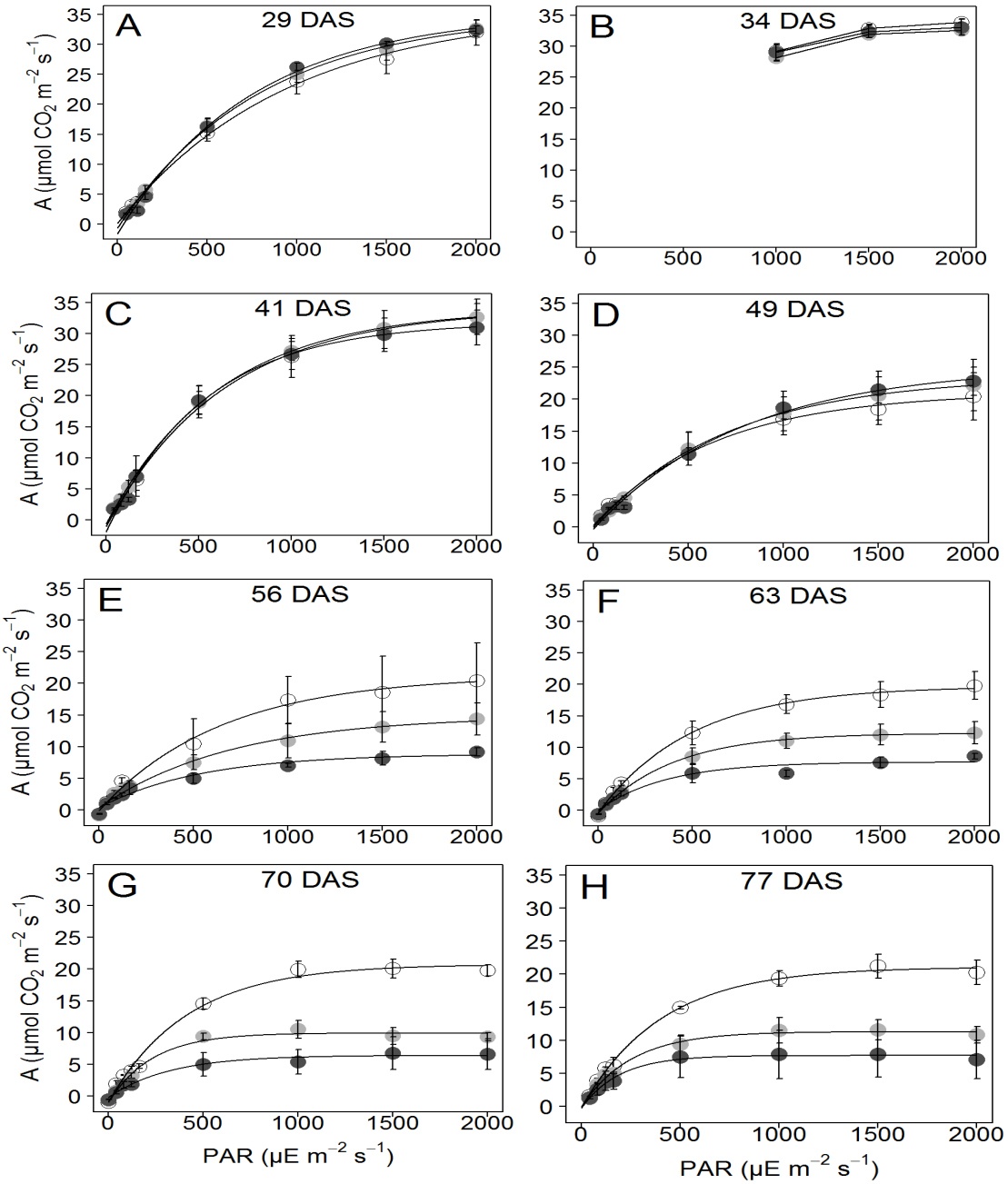

Figure 4. Time course (29-77 DAS) of photosynthesis-light response curves for leaves from rice plants infested by *R. fistulosa* compared with leaves from un-infested rice plants in Experiment 3. Grey circles: 6 *R. fistulosa* plants per pot; black circles: 13 *R. fistulosa* plants per pot; open circles: un-infested plants. Error bars represent standard errors of mean.

Table 1. Analysis of variance and means of photosynthesis parameters of leaves from single rice plants infested with three *R. fistulosa* densities (0, 6 and 13 parasites/pot) measured at different points in time (expressed in days after sowing, DAS) in Experiment 3. A_{max}= maximum gross assimilation rate (µmol CO_2 m^{-2} s^{-1}); A_{mass}= maximum photosynthetic rate per gram of leaf dry weight; ε =initial light use efficiency (µmol CO_2 μE^{-1}); R_d = dark respiration (µmol CO_2 m^{-2} s^{-1}); SLA= specific leaf area (m^2 kg^{-1}).

	DAS	29		41		49		56		63		70		77		Average		DAS	Density	DAS x Density
Amax	Un-infested	32.83	ab	35.16	a	21.38	cd	23.15	c	20.1	cd	21.77	cd	21.53	cd	25.13	*P*	**<.001**	**<.001**	**0.007**
	6 *R. fistulosa*/pot	34.32	a	34.67	a	24.57	c	14.89	de	13.95	de	11.03	e	11.67	e	20.73				
	13 R. fistulosa/pot	38.29	a	33.88	ab	25.88	bc	9.05	e	8.21	e	7.01	e	7.96	e	18.61				
	Average	35.15		34.57		23.94		15.7		14.08		13.27		13.72						
Amass	Un-infested	1.15	ab	1.04	b	0.69	cd	0.75	c	0.48	def	0.47	def	0.45	def	0.719	*P*	**<.001**	**0.03**	**0.04**
	6 *R. fistulosa*/pot	1.22	ab	1.08	b	0.78	c	0.50	de	0.39	efg	0.24	fg	0.25	fg	0.63743				
	13 R. fistulosa/pot	1.36	a	1.11	ab	0.77	c	0.31	efg	0.26	efg	0.18	g	0.17	g	0.59389				
	Average	1.24		1.08		0.74		0.52		0.38		0.30		0.29						
ε	Un-infested	0.04		0.06		0.04		0.04		0.04		0.05		0.06		0.047	x	*P*	**<.001**	**0.009**	0.136
	6 R. fistulosa/pot	0.05		0.06		0.04		0.03		0.03		0.05		0.05		0.044	x			
	13 R. fistulosa/pot	0.05		0.06		0.04		0.02		0.02		0.02		0.04		0.037	y			
	Average	0.05	B	0.06	A	0.04	C	0.03	D	0.03	D	0.04	C	0.05	B					
Rd	Un-infested	0.54		1.42		0.14		0.56		0.42		0.94		0.34		0.623	*P*	**0.002**	0.867	0.271
	6 R. fistulosa/pot	1.23		0.76		0.85		0.21		0.53		1.05		0.39		0.716				
	13 R. fistulosa/pot	1.64		1.97		0.49		0.14		0.11		0.37		0.26		0.711				
	Average	1.14	A	1.38	A	0.49	B	0.31	B	0.35	B	0.79	AB	0.33	B					
SPAD	Un-infested	35.8		39.08		31.67		28.65		31.1		32.8		35.72		33.55	*P*	**<.001**	**<.001**	**0.005**
	6 R. fistulosa/pot	34.67		39.52		34.27		26.62		30.88		28.7		31.7		32.34				
	13 R. fistulosa/pot	33.95		39.9		35.02		23.1		22.93		20.07		25.6		28.65				
	Average	34.81		39.50		33.65		26.12		28.30		27.19		31.01						
SLA	Un-infested	35.09	ab	29.59	def	32.12	bcd	33.57	abc	24.09	g	21.60	gh	20.93	h	28.14	*P*	**<.001**	**<.001**	**<.001**
	6 R. fistulosa/pot	35.46	a	31.13	cde	32.08	bcd	34.15	abc	27.89	f	21.35	gh	21.28	gh	29.05				
	13 R. fistulosa/pot	35.32	a	32.77	abc	29.34	def	34.47	ab	32.11	bcd	28.28	ef	23.06	gh	30.76				
	Average	35.29		31.17		31.18		34.06		28.03		23.74		21.76						

Impact of R. fistulosa infestation on stomatal conductance of rice

In Experiment 3, the stomatal conductance (mol $m^{-2}s^{-1}$) of un-infested plants and rice plants infested by 6 and 13 parasites per pot, was compared at different observations times (56, 63 and 70 DAS) and at different light intensities (PAR of 0-2000 μE $m^{-2}s^{-1}$). The stomatal conductance increased with increasing irradiance from 0 to the highest light intensity (PAR) of 2000 μE $m^{-2}s^{-1}$ (Fig. 5; Table S4). At 56 DAS the only significant difference between stomatal conductance of un-infested and parasite-infested plants was in the dark (at PAR=0; P=0.025) and at a light intensity (PAR) of 120 μE $m^{-2}s^{-1}$ (P=0.002). In the dark, values of infested plants were 37% and 39% less than that of un-infested plants, (at infestation rates of 6 and 13 parasites per pot resp.), while at 120 μE $m^{-2}s^{-1}$, the values of infested plants were 54% (0.0492) and 64% lower (0.0392) than that of un-infested plants (0.1074) (at infestation rates of 6 and 13 parasites per pot resp.). At 63 DAS, significant differences between stomatal conductance of infested and un-infested plants were only observed at irradiance levels of 1000 μE $m^{-2}s^{-1}$ PAR. Compared with un-infested plants (0.287), the stomatal conductance of infested plants was 34% lower (i.e., 0.188) on rice plants grown with 6 parasites per pot and 65% (i.e., 0.1015) lower on rice plants grown with 13 parasites per pot (P<0.001). At 70 DAS, significant differences between stomatal conductance of un-infested and parasite-infested plants were observed at irradiance levels of 40 μE $m^{-2}s^{-1}$ (P<0.01). Stomatal conductance of infested plants (i.e., 0.043 and 0.048 at 6 and 13 parasites per pot resp.), was 59% and 40% less than that of un-infested rice plants (i.e., 0.104). At PAR levels of 500 and above, stomatal conductance of rice plants infested by *R. fistulosa* plants were significantly reduced by 57-70% compared with un-infested plants (Fig. 6).

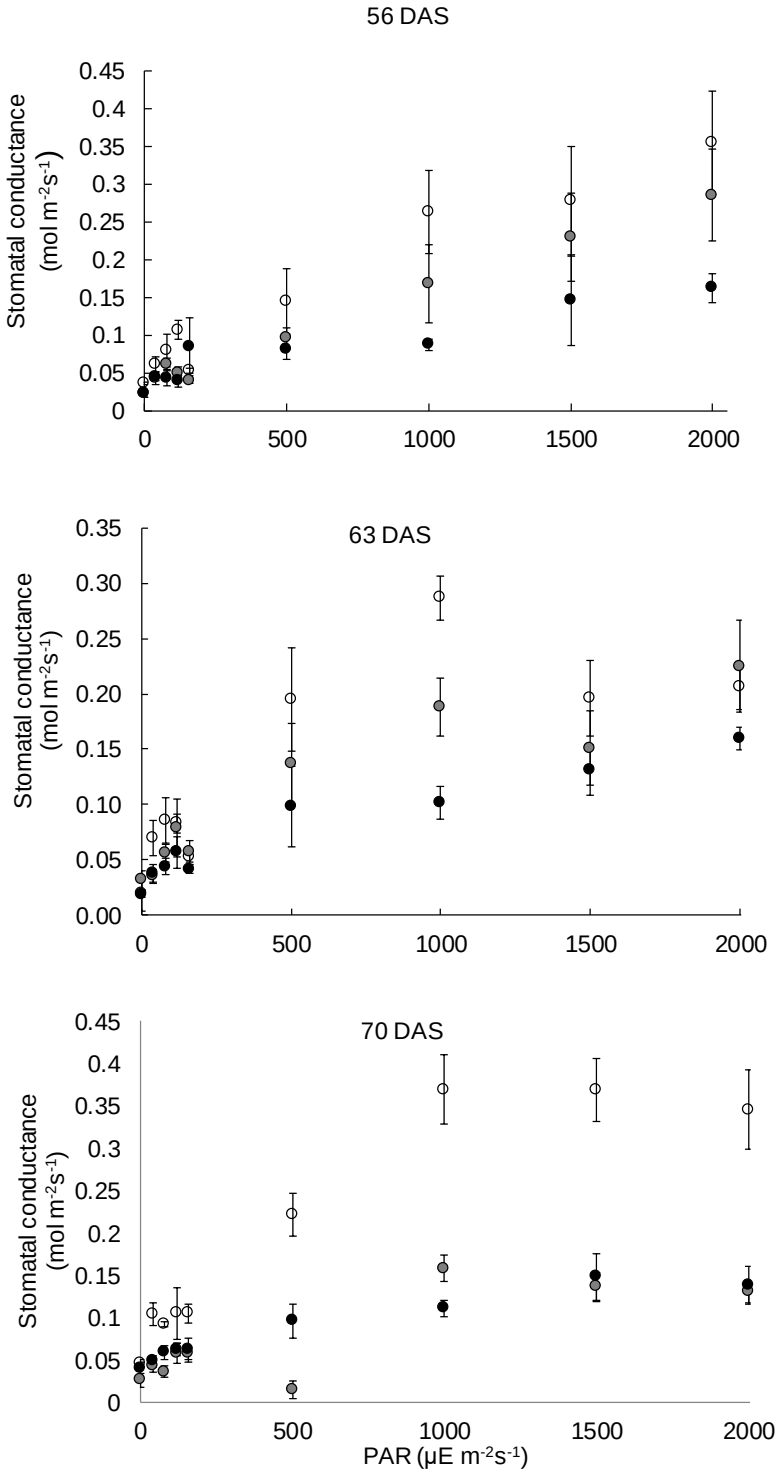

Figure 5. Stomatal conductance of un-infested rice plants and rice plants infested by 6 *R. fistulosa* plants per pot (infested 1) and 13 *R. fistulosa* plants per pot (infected 2) in Experiment 3 at 56, 63 and 70 DAS. Error bars are standard errors of mean.

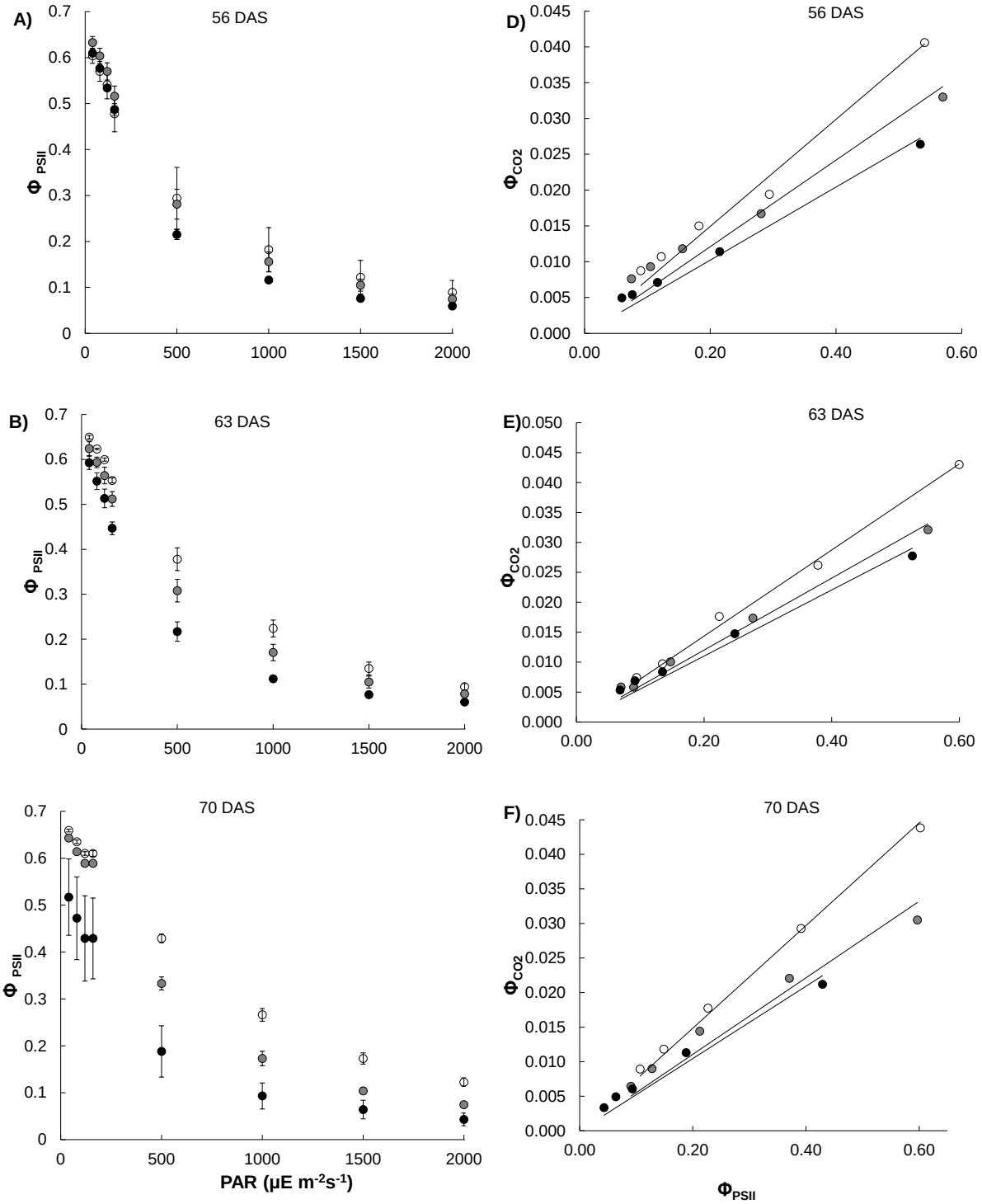

Figure 6. The light response of Φ_{PSII} (A, B, C) and the relationship between Φ_{CO2}, and Φ_{PSII} (E, F, G) of leaves of un-infested rice plants and *R. fistulosa*-infested plants at infection rates of 6 plants (infested 1) and 13 plants (infested 2) in Experiment 3. Error bars are standard errors of mean.

Light adapted quantum efficiency of PSII electron transport and quantum yield of CO_2 assimilation

The PSII electron transport Φ_{PSII}, indicates the efficiency for light use of photosystem II. In this study (Experiment 3), we found that the Φ_{PSII} of un-infested plants and rice plants infested by 6 and 13 parasites per pot, decreased with increasing irradiance from 0 to the highest light level

at 2000 μE $m^{-2}s^{-1}$ (Fig. 6; Table S5). At 56 DAS there was no significant difference between Φ_{PSII} of un-infested and parasite-infested plants. At 63 DAS, significant differences between Φ_{PSII} of infested and un-infested plants were observed at irradiance levels of 80, 120, 160, 500, 1000 and 2000 μE $m^{-2}s^{-1}$ PAR (Table S5). The Φ_{PSII} of plants infested with 6 parasites per pot (infested 1) were 5%, 6%, 7%, 24% and 17% lower than that of un-infested plants, at 80, 120, 160, 500, 1000 and 2000 μE $m^{-2}s^{-1}$, respectively. The Φ_{PSII} of plants infested with 13 parasites per pot (infested 2) were 12%, 14%, 19%, 50% and 36% lower than that of un-infested plants at the same light levels (Table S5). At 70 DAS, significant differences between Φ_{PSII} of infested and un-infested plants were observed at irradiance levels between 500 and 2000 μE $m^{-2}s^{-1}$ (Fig. 6C; Table S5). Compared with un-infested plants, the Φ_{PSII} of plant infested with 6 parasites per pot were 22%, 35%, 40% and 39% lower than un-infested plants at irradiance levels of 500, 1000, 1500 and 2000 μE $m^{-2}s^{-1}$ respectively. Similarly, as shown at 63 DAS, the effect was greater at infestation rates of 13 *R. fistulosa* plants where the reduction of Φ_{PSII} was 56%, 65%, 63% and 65% lower than un-infested plants at these light levels.

The quantum yield of CO_2 assimilation, Φ_{CO2}, was correlated with Φ_{PSII} for both the un-infested plants and the rice plants infested by 6 and 13 *R. fistulosa* plants at all three observation dates (Fig. 6D-F). In all treatments, a linear relationship was observed between Φ_{CO2} and Φ_{PSII} (R^2 values: 0.938 - 0.996). Compared with un-infested plants, the slope of this linear relation was reduced for infested plants at both infestation levels, indicating that with the same amount of available electrons less CO_2 was fixed, and hence CO_2 fixation was relatively less efficient. Depending on observation time and infestation level, this parasite-induced reduction in slopes ranged from 16 to 32%.

DISCUSSION

All three experiments clearly showed that infection with the facultative parasitic plant *Rhamphicarpa fistulosa* negatively affects the leaf photosynthetic rate of a rice host plant. In Experiment 1 and 3, where leaf photosynthetic rate of rice was observed at weekly intervals, the first parasite-induced reductions in photosynthetic rate only appeared at around eight weeks after sowing. Exhibition of this effect started with a reduced maximum photosynthesis (A_{max}), at around 56 DAS and was followed by reductions in initial light use efficiency (ε), about 9 days later. A previous study has shown that expression of relative *Rhamphicarpa*-induced reductions in host growth rates started around the same time (Kabiri et al., 2016), whereas the parasite started to benefit from the host much earlier, at 42 DAS (Kabiri et al., 2017). These reductions in photosynthesis were stronger at later observation times and with an increased number of parasite plants. In Experiment 3, where parasite infestation level was relatively low, the reductions in initial light use efficiency were only significant at the highest infestation level. Obligate parasites, such as *Striga hermonthica*, cause similar reductions in A_{max} and ε of the host plant but these are expressed at much earlier stages, i.e. around 19 days after emergence (DAE) of the host plant seedling (e.g. van Ast, 2006). The apparent delay in parasite-induced effects on host plant photosynthesis may be caused by the inherent delay in establishment of a host-parasite relation observed with facultative parasites, estimated to occur around 50 DAS (Kabiri et al., 2017). For *S. hermonthica*, the first attachments of *S. hermonthica* to the roots of the host are generally observed from10 DAE onwards (e.g. van Ast, (2006).

The reduction in A_{max} of parasite infested rice plants compared to un-infested plants generally increased with parasite infestation level, but not proportionally. Beyond the second infestation level (14 seeds per pot), the A_{max} levels as well as the light use efficiencies stabilised. Similar non-linear relationships between parasite infection level and host performance have been observed in a range of other parasite-host associations. For example, the effects of stem holoparasites (Shen et al., 2011) obligate and facultative root parasites (Gurney et al., 1999; Puustinen and Salonen, 1999) on host performance was also found to plateau once a certain

parasite density was reached. This indicates a decreasing impact on host photosynthesis per parasite with increasing infection levels. Such density dependency could be related to intraspecific competition (i.e., between parasite individuals) leading to reductions in biomass per parasite with increasing numbers of infections. A previous study found a reduction in average plant height and biomass of *R. fistulosa* with increasing parasite infestation levels (Kabiri et al., 2016). This phenomenon was observed whether the parasite grew in the presence or absence of a host.

The maximum reduction in A_{max} was about 60%, and this was attained at 65 DAS or beyond (depending on infestation level and experiment). In comparison with the association between rice and an obligate hemi-parasite, *Striga asiatica*, these negative effects on host photosynthesis were moderate and occurred late (Rodenburg et al., 2017). It was observed that induced reductions in leaf photosynthesis of rice to range between 60 and 100%, depending on genotype, and these high reductions were already obtained at 45 DAS (Rodenburg et al., 2017). In a study on the holo-parasitic *Orobanche ramosa*-tomato association (Mauromicale et al., 2008) photosynthesis of tomato was suppressed by 39-50%. Reductions in photosynthetic rates per unit leaf area can be partly caused by thinner leaves and reduced chlorophyll content. There was an increase in SLA (thinner leaves) and a reduction in SPAD (chlorophyll content) values of leaves of infested rice plants in all experiments. However, whereas the photosynthesis was affected at both infestation levels in Experiment 3, from 56 DAS onwards, the parasite effect on SPAD, and to a lesser extent SLA, was more consistent at the high parasite infestation level compared to the low infestation level and were also expressed at a later observation date. This points to a lower parasite effect on leaf-based parameters but could indicate underlying influences of additional mechanisms that impact host photosynthesis. As the deviations in SPAD values mirrored that of the SLAs, the observed reduction in leaf chlorophyll content resulted at least partly from leaves getting thinner. It cannot be determined whether the chlorophyll content at cellular level was also affected because of parasite infection.

The mechanisms behind parasite suppression of host photosynthesis are complex but have frequently been associated with a lowering of stomatal conductance (Frost et al., 1997; Gurney et al., 1995). Lower stomatal conductance reduces the diffusion of CO_2 into the leaves (Farquhar and Sharkey, 1982), and this feature was previously found in *Striga*-infested sorghum (Frost et al., 1997; Gurney et al., 1995). Parasitic plants generally have high transpiration rates, which may result in water stress at the host level, resulting in a closure of stomata (Stewart and Press, 1990). Some root parasites, such as nematodes, are known to reduce transpiration rates of their host (Schans and Arntzen, 1991) and it has been suggested that this is a result of increased levels of abscisic acid (Chen et al., 2011). Abscisic acid (ABA) is known as a stress hormone and it can cause stomata closure also in the absence of water shortage. In parasitic plant – host plant associations, Taylor et al., (1996) found indications that ABA could be involved in inhibiting stomatal conductance in *Striga*-infected maize plants, while Chen et al., (2011) observed an increase in host ABA following infection of *Mikania micrantha* by the holo-parasite *Cuscuta campestris*. This contributed to a reduction in host stomatal conductance, transpiration rates and net photosynthetic rates of the host. ABA can also be involved in reducing leaf area expansion and stem extension, and in increasing the root:shoot ratio, all of which are symptoms of parasitic plant infections (Frost et al., 1997; Taylor et al., 1996; Watling and Press, 2001). These morphological phenomena are also observed in rice plants after infection with *R. fistulosa* (Kabiri et al., 2017), suggesting that also in this host-parasite association ABA concentrations might be affected.

Some known mechanisms include reduced efficiencies in the photosynthetic apparatus of host leaves. It has been shown that, under suppressed photosynthesis, the photon flux density (PFD) to photosynthesis ratio increases such that excess absorbed light beyond that utilized by photosynthesis damages the photosynthetic apparatus (Demmin-Adams and Adams, 1992). The

rate of photodamage to PSII occurs when PSII is completely inhibited and it has been observed that at this point, the rate of gross photodamage to PSII is proportional to the level of incident light (Tyystjärvi and Aro, 1996). In these circumstances the inhibition of electron transport and the interruption of the Calvin cycle do not affect the rate of photodamage to PSII (Allakhverdiev et al., 2005; Hakala et al., 2005; Nishiyama et al., 2004; Takahashi and Murata, 2005). However, it is still debatable whether photodamage to PSII can at least be partly attributed to the effects of excessive light energy on the PSII reaction center (Takahashi and Badger, 2011; Tyystjärvi, 2008; Vass and Cser, 2009). After photodamage to PSII, the impaired PSII proteins are substituted with freshly manufactured proteins following partial disassembly of the PSII complex in a process called the 'PSII repair cycle' (Aro et al., 2005). The rate of PSII repair relies on the presence of light, but is saturated at rather low light intensities (Allakhverdiev and Murata, 2004). However, when there is excess light for photosynthesis, the rate of repair is reduced because of inhibition of the synthesis of the proteins. This indicates that abiotic or biotic stressors that limit the Calvin cycle activity directly or indirectly through stomatal closure, can cause inhibition of PSII repair (Murata et al., 2007; Takahashi and Murata, 2008). Parasite effects on hosts are such stressors. For example, *Striga*-infested plants were found to be more sensitive to light-induced reduction in the photosynthetic capacity (photoinhibition) because of damage to the electron transport system (Ramlan and Graves, 1996; Rodenburg et al., 2008). Consequently, the recovery of the apparent quantum yield was slower amongst infested plants. In this study, the chlorophyll fluorescence data on PSII measured at 56, 63 and 70 DAS do provide clues on how photosynthesis was affected after infection with *R. fistulosa*. We found significant reductions in PSII of infested rice plants compared with un-infested plants. Similar observations were made by Cameron et al., (2008) with another facultative parasitic plant, *Rhinanthus minor*, parasitizing on the wild grass species *Phleum bertolinii*. Our results show that in addition, reduced electron transport rates could also stem from decreased intracellular CO_2 concentrations, as the parasite has also shown to cause reductions in host stomatal conductance.

The large impact of *R. fistulosa* infection on both stomatal conductance and photosynthetic metabolism of rice is similar to the effect that other parasitic plant species have on C_3 hosts. Also in the *Striga*-C_3 host associations (Watling and Press, 2000) and in *Cuscuta*-host associations (Shen et al., 2007) the parasite impaired both stomatal conductance and host photosynthetic metabolism. In the same association between *C. campestris* and its host *M. micrantha*, Shen et al., (2011) found that soluble protein concentrations were lower in the host *M. micrantha* when infected by more than two *C. campestris* parasites. In C_3 plants, rubisco is the most abundant soluble protein found in the leaves and is the most important enzyme that fixes CO_2 in C_3 photosynthesis (Evans, 1989; Parry et al., 2003). Reducing Rubisco has been linked to reduction of photosynthesis rates (Furbank et al., 1996; Stitt and Schulze, 1994). The current study showed that the slope that represents the relation between Φ_{PSII} and Φ_{CO2} was significantly reduced. A reduction in the ratio Φ_{CO2}/Φ_{PSII} could mean that there were more electrons passing through PSII than were required to sustain the observed CO_2 assimilation. Possibly, alternative electron sinks other than CO_2 assimilation were operating. Fryer et al. (1998) suggested several alternative sinks to cause low Φ_{CO2}/Φ_{PSII} values, including photorespiration. Another explanation for the reduced Φ_{CO2}/Φ_{PSII} ratio of infested plants is that absorption of PAR was reduced, as our Φ_{CO2} was based on incident radiation. Absorption of PAR was not directly measured. If infection resulted in a lowered irradiance absorption, it means that the actual efficiency of CO_2-fixation was underestimated. This in turn will contribute to a reduction in the slope of the relation between Φ_{PSII} and Φ_{CO2}, as observed here. The reduced SPAD-values of infested leaves indeed provide support for the suggestion that a reduced absorption of PAR contributed, at least in part, to the reduced efficiency of electrons for fixing CO_2.

Conclusions

Our results clearly show that the facultative parasitic plant *Rhamphicarpa fistulosa* impacts on the photochemical process and gas exchange of its host. Infection led to significant reductions in photosynthetic rate, stomatal conductance, the quantum efficiency of PSII electron transport (Φ_{PSII}) and chlorophyll content, but not dark respiration. Reductions were observed starting from eight weeks after sowing, which is much later than with obligate hemi-parasitic plant infection. Moreover, there was a considerable time lag between the parasite's acquisition of benefits from the association, in terms of growth (around 42 DAS), and the reduction of host photosynthesis (around 56 DAS). The timing of the relative suppression of host photosynthesis observed in this study coincided with the start of the expression of relative reductions in host growth rates that were observed previously. This indicated that *R. fistulosa* affects host growth by first extracting assimilates and making considerable gains in growth, before impacting host photosynthesis. Except for dark respiration rates, which were never affected by parasite infection, suppression of host photosynthesis at light saturation, the initial light-use efficiency, chlorophyll content, specific leaf area and shoot weight were parasite density dependent with a stronger effect for higher parasite densities. In addition, parasite infection led to a reduced ratio of quantum yield of CO_2 assimilation (Φ_{CO2}) to quantum efficiency of PSII (Φ_{PSII}). These results shed the first light on the metabolic host-parasite interactions between rice and the facultative parasitic plant *R. fistulosa*.

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