Photosynthetic Diffusional Constraints Affect Yield in Drought Stressed Rice Cultivars during Flowering

Marco Lauteri1, Matthew Haworth2*, Rachid Serraj3,4, Maria Cristina Monteverdi5, Mauro Centritto2

1 Institute of Agro-Environmental and Forest Biology, National Research Council, Porano, Italy, 2 Trees and Timber Institute (IVALSA – Istituto per la valorizzazione del legno e delle specie arboree), National Research Council, Florence, Italy, 3 Consultative Group on International Agricultural Research, Independent Science and Partnership Council Secretariat, Food and Agriculture Organisation of the United Nations, Rome, Italy, 4 International Rice Research Institute, Los Banos, Philippines, 5 Forestry Research Centre, National Research Council, Arezzo, Italy

Abstract

Global production of rice (Oryza sativa) grain is limited by water availability and the low ‘leaf-level’ photosynthetic capacity of many cultivars. Oryza sativa is extremely susceptible to water-deficits; therefore, predicted increases in the frequency and duration of drought events, combined with future rises in global temperatures and food demand, necessitate the development of more productive and drought tolerant cultivars. We investigated the underlying physiological, isotopic and morphological responses to water-deficit in seven common varieties of O. sativa, subjected to prolonged drought of varying intensities, for phenotyping purposes in open field conditions. Significant variation was observed in leaf-level photosynthesis rates (A) under both water treatments. Yield and A were influenced by the conductance of the mesophyll layer to CO2 (gm) and not by stomatal conductance (gs). Mesophyll conductance declined during drought to differing extents among the cultivars; those varieties that maintained gm during water-deficit sustained A and yield to a greater extent. However, the variety with the highest gm and yield under well-watered conditions (IRSS419-04) was distinct from the most effective cultivar under drought (Vandana). Mesophyll conductance most effectively characterises the photosynthetic capacity and yield of O. sativa cultivars under both well-watered and water-deficit conditions; however, the desired attributes of high gm during optimal growth conditions and the capacity for gm to remain constant during water-deficit may be mutually exclusive. Nonetheless, future genetic and physiological studies aimed at enhancing O. sativa yield and drought stress tolerance should investigate the biochemistry and morphology of the interface between the sub-stomatal pore and mesophyll layer.

Introduction

Global production of rice (Oryza sativa) is limited by the availability of freshwater [1] and the tolerance of rice cultivars to drought stress [2]. Nonetheless, O. sativa is cultivated in a diverse range of climates and habitats, with lowland varieties generally considered to be more productive, but less tolerant of drought, than their upland counterparts [3]. Selective breeding of O. sativa varieties has increased the grain production of individual plants by 70% since 1965 [4]. However, these increases in productivity have been restricted by the comparatively low ‘leaf-level’ photosynthetic capacity of O. sativa relative to other C3 crops [5,6]. Phenotypic screening of the photosynthetic and gas-exchange characteristics of O. sativa varieties under water deficit conditions may permit the identification of attributes that confer both drought tolerance and high productivity [7,8].

Yield in varieties of O. sativa are closely related to photosynthesis under both well-watered and drought conditions [7,9]. The uptake of CO2 for photosynthesis involves two major resistance steps at the stomata and at the surface of the mesophyll layer [10,11]. Stomatal (gs) and mesophyll conductance (gm) to CO2 often decrease in unison in response to environmental stresses [7,12–14]. Upland and lowland O. sativa cultivars exposed to a soil moisture gradient ranging from high water-availability to severe water-deficit exhibited identical reductions in both gs and gm that correlated with the rate of photosynthesis (A) and yield. Most notably, while there was no observable difference in A, gs or gm response to drought between the upland and the lowland varieties, those cultivars that exhibited the highest values of total conductance to CO2 (g: where g = [gs * gm] / [gs + gm]) supported higher A and yield under all levels of water availability [7]. Those O. sativa varieties with higher capacities for CO2 uptake are therefore the most likely to offer the greatest potential improvement of leaf-level photosynthesis towards achieving enhanced yields [9,15,16]. However, an increase in the capacity for leaf gas


Editor: Wagner L. Araujo, Universidade Federal de Vícosa, Brazil

Received May 20, 2014; Accepted September 1, 2014; Published October 2, 2014

Copyright: © 2014 Lauteri et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

Funding: This work was funded by: Ministero dell’Istruzione, dell’Università e della Ricerca of Italy (PRIN grant: “Going to the root of plant productivity: how the rhizosphere interact with the aboveground armament for indirect and direct defense against abiotic and biotic stressors (PRO-ROOT)” URL: http://hubmiur.pubblica.istruzione.it/web/universita/home and Marie Curie Intra European Fellowship (2010-275626) - HAWORTH - Evolutionary adaptation to atmospheric carbon dioxide, URL: http://ec.europa.eu/research/mariecurieactions/about-mca/actions/ief/index_en.htm. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: matthew.haworth@hotmail.com
exchange may be accompanied by reduced drought tolerance [17,19].

These differences in the photosynthetic and leaf gas-exchange capacities of O. sativa varieties have been ascribed to genetic variation [9]. The quantitative trait loci responsible for leaf gas-exchange and photosynthetic parameters in O. sativa tend to be found in adjacent regions of the plant genome; indicative of the exertion of selective pressures favouring coordination and high heritability of photosynthetic behaviour [19,20]. Therefore, effective screening and analysis of the photosynthetic and gas exchange characteristics of O. sativa varieties under stress conditions has the potential to identify functional traits associated with optimisation of water use efficiency (WUE) and the maintenance of yield and A under drought [21]. Rates of photosynthesis are not only determined by diffusive CO$_2$-uptake, but also by the activity of ribulose-1,5-bisphosphate (RubisCO) [22]. However, exposure of thirteen hydroponically grown O. sativa varieties to physiological drought did not suggest that the capacity of RubisCO carboxylation (V$_{\text{cmax}}$) or the maximum rate of electron transport required for ribulose-1,5-bisphosphate regeneration (J$_{\text{ecmax}}$) were associated with A or grain yield. Rather, A and yield were largely determined by g$_s$ and g$_{m}$, setting the limits for diffusional CO$_2$ uptake [9], consistent with field based studies [7]. Furthermore, the intrinsic water-use efficiency (A/g)$_s$ of the O. sativa varieties was closely related to the ratio of g$_{m}$ to g$_s$ [9], and this attribute showed the greatest variability between cultivars, and thus potential in terms of possible improvements in yield and growth through maximising WUE [5,9,21]. In theory, those varieties with high g$_{m}$ to g$_s$ ratios should be most suited to maintaining photosynthesis and yield under drought stress conditions, while high values of both g$_{m}$ and g$_s$ should be favourable for varieties grown under constant high water availability [21].

The observation that A and yield of O. sativa closely correlate to g$_{m}$ [7,9,16] has led to a focus on the interface between the leaf mesophyll cells and internal sub-stomatal air space in the improvement of rice productivity [23]. Adachi et al. [16] crossed two varieties of O. sativa to produce a new cultivar with higher values of g$_{m}$, which were associated with modifications of the mesophyll structure that increased the surface area available for CO$_2$ uptake; thus demonstrating the potential for enhanced productivity through increased g$_{m}$. In addition to morphological adaptations [15], differences in g$_{m}$ between O. sativa varieties are also likely affected by biochemical factors [23] such as the abundance of coporin proteins responsible for active transport of CO$_2$ [24,25] (Kaldenhoff, 2012). Furthermore, the abundance and activity of these proteins has been observed to decline under water-stress [26]. Exposure to drought is associated with declines in values of A, g$_s$, and g$_{m}$ [11,27]. Sustained impairment of A following the alleviation of drought stress is commonly related with continued biochemical constraints to the uptake and assimilation of CO$_2$ [28]. However, experimental evidence suggests that O. sativa does not display prolonged reductions in A following the cessation of drought, and that the observed constraints to A under water-stress are largely physical impediments to CO$_2$ diffusion [7,9,29,30]. This suggests that artificial selection of those O. sativa varieties with high g$_{m}$ would enhance A while not negatively affecting drought tolerance or recovery.

Rice production is sensitive to water deficit at different developmental stages of plant growth. For example, early drought stress can affect leaf expansion [3], and later drought stress during flowering or grain-filling stages can disrupt grain formation and development [31,32]. The photosynthetic and yield responses of seven O. sativa varieties (five upland and two lowland varieties chosen to represent genetic variability) were analysed in response to sustained drought. As different O. sativa varieties have different phenologies (for example Van forms flower inflorescences relatively earlier than Moro), through the use of a drip irrigation system it was possible to apply the stress at the same developmental stage for each variety. This has the advantage over a previous study into the drought tolerance of O. sativa varieties conducted at the IRRI in that all varieties experienced drought at the same phenological stage, therefore eliminating a crucial source of error whereby early flowering varieties outperformed late developing cultivars [cf. 7]. Mesophyll conductance to CO$_2$ is of key interest to the productivity and drought tolerance of O. sativa [7,16], however it is not possible to directly measure g$_{m}$ and due to the uncertainties associated with its calculation [33] (partially in the use of the variable J method under stress conditions) it is preferable to simultaneously use two independent methodologies in the estimation of g$_{m}$ [34]. We therefore employed two independent methodologies in the estimation of g$_{m}$: firstly, based upon the synchronous measurement of leaf gas exchange and chlorophyll fluorescence parameters (variable J), and secondly, the analysis of carbon isotope discrimination ($\Delta^{13}C$) in recently formed metabolites to assess their effectiveness in an analysis of O. sativa drought tolerance in the field. Further details of the variable J and ‘$\Delta^{13}C$ of recently synthesised sugars’ methods of g$_{m}$ analysis are presented in the Material and Methods section. This study aimed to: 1) assess the effect of g$_s$ and g$_{m}$ on A and yield in well-watered and drought conditions; 2) analyse differences in g$_{m}$ calculation between the ‘variable J’ [35] and ‘$\Delta^{13}C$ of recently synthesised sugars’ [15] methods; and, 3) identify O. sativa varieties that exhibit drought tolerance and/or high productivity, and may be suitable for further genomic/biochemical development to further enhance grain production.

Materials and Methods

Plant material and growth conditions

The experiment was conducted at the International Rice Research Institute Experimental Station at Los Banos, Philippines (14° 11’N 121° 15’E, 21 m above sea level) during the dry season (January to May). Rainfall was minimal, however, temperature and leaf to air vapour pressure difference (VPD) increased during the experimental period. The soil at the experimental site is a neutral Andaqueptic Haplauquot (pH = 6.5). Seven O. sativa cultivars were studied: Apo, IR53419-04 (IR55), IR64, IR71253-19-1 (IR71), Moroberekan (Moro), PSBRc80 (PS80) and Vandana (Van). Moroberekan failed to set or produce grains and seemed to suffer from disease and heat sensitivity, particularly under drought conditions and it was not possible to record physiological measurements for this variety under water deficit stress conditions. Seeds were sown in dry soil at a rate of 80 kg ha$^{-1}$ in rows spaced ~25 cm apart. Fertilisation with nitrogen was conducted on two occasions at application rates of 90 and 120 kg ha$^{-1}$, alongside fertilisation of 30 kg ha$^{-1}$ of phosphate and potassium. Weeds were controlled by the application of standard agricultural herbicides and manual plucking. Pesticides were used to control insect pests; in particular stem borers.

Experimental plots for each O. sativa variety measured 5 m x 2 m, with adjacent irrigated and drought-stressed treatments. The plots were arranged in a randomized block design. A sprinkler system was initially used to provide equal irrigation levels to all plants until flower (panicle) development, when the water deficit treatment was instigated. During the drought stress period, the well-watered treatments were irrigated by use of a drip irrigation system, with soil water potential maintained...
above -0.04 MPa (monitored using tensiometers placed at soil depths of 15 and 30 cm) – the level at which the more sensitive rice cultivars begin to exhibit a reduction in transpiration [36].

**Leaf gas exchange measurements**

Leaf gas-exchange and fluorescence of plants grown under both well-watered treatment or stress conditions were simultaneously measured with a LI-6400-40 leaf chamber fluorometer (Li-Cor, Inc., Nebraska, USA) on flag leaves enclosed in a temperature, light and humidity controlled cuvette. The measurements were made in situ between 11.00 and 15.00, in saturating PPFD (1400 μmol m⁻² s⁻¹), with relative humidity ranging between 45–55%, and a leaf temperature of 30°C. Instantaneous measurements of steady-state A, gᵣ, internal [CO₂] (Cᵢ) and the quantum yield of PSII in the light (ΔF/Φm) were made on six to 14 plants per treatment. Measurements of dark respiration (R_d) were also made at ambient CO₂ concentration in the dark on the same leaves. Instantaneous transpiration efficiency was calculated as the ratio of A to gₑ.

**Carbon isotope discrimination**

Three of the same flag leaves used for in situ gas exchange measurements were collected in the evening, oven dried at 70°C until their weight remained constant and were then stored until soluble sugar extraction, following the protocol of Richter et al. [37]. Leaves were finely ground in liquid nitrogen and shaken for 60 min in water at room temperature. After centrifugation (15 min at 5000 g), the supernatant was sequentially mixed with cationic (Dowex-50) and anionic (Dowex-1) exchange resins. The residual solution of purified soluble sugars was freeze-dried and carbon isotope composition (δ₁³C) determined using a mass-spectrometer. Additional isotope ratio mass spectrometry analyses were performed on the pellet samples that remained after the extraction of soluble sugars. Samples of approximately 1 mg were used for δ₁³C analysis. Stable isotope ratios were determined using a continuous-flow triple-collector isotope ratio mass spectrometer (ISOPRIME, GV, Manchester, UK). Solid samples were quantitatively combusted in an elemental analyser (Model NA 1500, Carlo Erba, Milan, Italy) and CO₂ was transferred in helium flow to the mass spectrometer. Calculations of carbon isotope discrimination (Δ₁³C) of solid samples were undertaken following the protocol of Farquhar et al. [38], assuming the carbon isotopic composition of CO₂ in air (δₑ) to be −8.0‰.

**Estimation of mesophyll conductance**

Mesophyll conductance to CO₂ diffusion is the inverse of the total resistance encountered in the transport of CO₂ across the leaf mesophyll. Estimation of gₘ is subject to a number of assumptions and sources of error, and it is preferable to employ two independent approaches in its calculation [34,39]. In this study gₘ was calculated using the ‘variable J’ and Δ₁³C of recently synthesized sugars methods. The calculation of gₘ using the variable J method is based on simultaneous measurements of gas-exchange and chlorophyll fluorescence parameters as described by Harley et al. [35] and Loreto et al. [40] (equations 1 and 2):

\[
gₘ = \frac{A}{C_l - \frac{\frac{F}{\Phi_m} - \frac{\Delta F}{\Phi_m} + \xi (A + R_d)}{J - \frac{\Delta F}{\Phi_m} + \xi (A + R_d)}} \tag{1}
\]

where the electron transport rate \(F/\Phi_m\) is calculated from fluorescence [41]:

\[
J_F = PPFD \times \frac{\Delta F}{\Phi_m} + \alpha \times \beta
\]

. Where \(F_m\) is the fluorescence maximum and the partitioning factor \(\beta\) between photosystems I and II was considered to be 0.5 and leaf absorbance \(\alpha\) was measured directly [42]. The variable \(J\) method is sensitive to the estimation of the CO₂ compensation point to photorespiration \(\Gamma^*_p\) and \(R_p\). For the results of this study to be directly comparable with those of Centritto et al. [7], the value of \(\Gamma^*_p\) used in the gas exchange algorithm was calculated using the Rubisco specific factor for an annual herb [43], consistent with previously published \(O. Sativa\ \Gamma^*_p\) values [44]. As measurements were conducted under field conditions it was not feasible to produce robust measurements of respiration in the light \(R_l\); respiration in the dark was therefore used in the calculation of \(g_m\). Gas exchange measurements were performed at ambient \([CO₂]_d\) concentration in the dark on the same leaves by switching off the light in the leaf cuvette; when CO₂ release from the leaf had become stable for approximately five to ten minutes this was recorded and considered to represent \(R_d\). To calculate the values of \(J\) determined by fluorescence \(J_F\) and gas-exchange \(J_l\), measurements were also conducted under non-photorespiratory conditions using 1% \([O₂]\) [35,40]. Electron transport rate and \(R_d\) values are presented in Figures S1 and S2. Total conductance to CO₂ \(gₘ\) was calculated as \(gₘ = gₑ \times g_m/(gₑ + g_m)\). The concentration of CO₂ at the chloroplast site \(Cᵢ\) was then calculated from mesophyll conductance values [46].

The method described by Lauteri et al. [47] for ‘on-line’ determinations was adapted to calculate \(g_m\) using the \(Δ₁³C\) of recently synthesized sugars [48]. Discrepancies between \(Δ₁³C\) of leaf soluble carbohydrates \(\Delta_{obs}\) and \(Δ₁³C\) expected on the basis of gas-exchange measurements \(\Delta_{exp}\) allow the estimate of \(g_m\) utilising the approach of Evans et al. [49] that was subsequently modified by Lloyd et al. [50]:

\[
g_m = \frac{(h - b_1 - x_1) + \alpha J/F}{\Delta_{exp} - \Delta_{obs}} = (J/F/Cᵢ) \tag{3}
\]

Where: \(b\) is the discrimination associated with carbohydrates reactions, taken to be 27.5‰; \(b\) is the fractionation occurring when CO₂ enters solutions (1.1‰ at 25°C); \(a\) is the fractionation during diffusion in water (0.7‰); \(f\) is the fractionation associated with photorespiration, and \(\rho\) is the partial pressure of CO₂ in air. Two \(g_m\) calculations were performed and compared, since two values were taken into account for the fractionation factor \(f\): 7% [50] and 0‰ [48,51]. The closest relationship between \(g_m\) estimates \(R^2 = 0.911\) using the variable J and \(Δ₁³C\) of recently synthesized sugars methods in all varieties was found when using a \(f\) value of 0‰.

**Harvesting**

Plants were harvested at physiological maturity by sampling 1 m of the 2 central rows of each plot. Data collected included biomass, plant height, anthesis date, tiller number, straw production, grain yield, and harvest index (HI).
Statistical analysis

Analyses of variance were conducted using SAS to generate least squares means for each entry. Leaf gas exchange data were tested using a simple factorial ANOVA (three-way maximum interactions) and, where appropriate, the treatment means of leaf properties and gas exchange parameters were compared using a Tukey post-hoc test.

Results

Drought stress induced reductions in yield, 4, and rates of gas-exchange across all the O. sativa varieties analysed in this study. The duration of time required for 50% of plants to develop flowers (flower days) did not correlate with yield in well-watered plants, but those varieties with a shorter flowering day period exhibited enhanced yield under drought stress (Fig. 1a). Varieties with shorter flowering days allocated a higher proportion of their total biomass to yield (harvest index: HI) under control and drought treatments (Fig. 1b). Positive allometric correlations between plant height and both yield (Fig. 1c) and HI (Fig. 1d) were observed, both under watered or drought conditions.

The carbon isotopic composition of the leaves of the O. sativa varieties became increasingly enriched in the heavier 13C isotope following water-stress, with the exception of IR64. Changes in the carbon isotopic composition of soluble sugars induced by the drought treatment were less consistent, with three varieties exhibiting increases and four decreases in the ratio of stable carbon isotopes (Table 1). Despite the lack of genotype x water treatment interaction, the varieties yielded differences in the ‘long-term’ isotopic signal of the bulk leaf material; some genotypes showed wide variation (up to –1.5% in pellets of Van) while others were more negligible (e.g. 0.2% in pellets of IR64). Drought caused also different variation in Δ13C of soluble sugars: up to –3.5% in PS80 but 2% in IR71. Under both well-watered and rainfed conditions, soluble sugars showed generally lower values of carbon isotopic discrimination when compared with the average Δ13C values of the flag leaf pellets. Significant differences were observed in the carbon isotopic discrimination values of leaf pellets between varieties, however this was not replicated in the analysis of soluble sugars (Table 1).

A strong positive correlation was observed between the two estimates of $g_{\infty}$ in the O. sativa varieties grown under control and drought conditions ($R^2 = 0.930; P<0.01$) (Fig. 2), excluding IR64 and PS80. Indeed, the lowland varieties IR64 and PS80 grown under well-watered conditions exhibited high values of $g_{\infty}$ derived from the application of the isotopic methodology. It was therefore decided to utilise $g_{\infty}$ measurements resulting from the variable J method in the subsequent analyses [an extended discussion of the contrasting merits of the approaches is given in 7]. Rates of $A$, $g$, and $g_{\infty}$ all showed consistent declines under water-stress conditions (Fig. 3). IR55 showed the highest yield and $g_{\infty}$ under well-watered conditions. Variety IR71 exhibited the highest general

Figure 1. The performance of seven rice varieties under well-watered (open symbols) and drought conditions (closed symbols): a) relationship between yield and flowering days (period of time for 50% of plants to develop flowers) under full-water (linear regression: $R^2 = 0.0150; \hat{F}_{1,4} = 0.0609; P = 0.817$) and drought (linear regression: $R^2 = 0.749; \hat{F}_{1,5} = 14.905; P = 0.0119$); b) allometric relationship between yield and plant height (linear regression: $R^2 = 0.637; \hat{F}_{1,10} = 17.536; P = 0.00187$); c) relationship between harvest index (HI: dry weight of grain relative to dry total plant biomass) and flowering days under full-water (linear regression: $R^2 = 0.637; \hat{F}_{1,5} = 8.760; P = 0.0315$) and drought (linear regression: $R^2 = 0.752; \hat{F}_{1,5} = 15.144; P = 0.0115$), and; d) relationship between HI and plant height (linear regression: $R^2 = 0.430; \hat{F}_{1,10} = 7.548; P = 0.0206$). Error bars indicate one standard error either side of the mean. Numbers next to data points indicate Oryza sativa variety: 1 = Apo; 2 = IR55; 3 = IR64; 4 = IR71; 5 = Moro; 6 = PSBRc80; 7 = Van.

doi:10.1371/journal.pone.0109054.g001
Table 1. Carbon isotope discrimination (%13C) of the flag leaf pellet (bulk) and soluble sugars of seven rice cultivars grown under drought and well-watered conditions in %.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Habitat</th>
<th>Bulk Leaf %13C(%)</th>
<th>Well-watered Mean</th>
<th>Drought Mean</th>
<th>Well-watered Drought Mean</th>
<th>Well-watered Drought Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo</td>
<td>U</td>
<td>21.6±0.6</td>
<td>18.5±0.9</td>
<td>18.6±0.6</td>
<td>17.4±0.5</td>
<td>17.1±0.5</td>
</tr>
<tr>
<td>IR55</td>
<td>U</td>
<td>21.4±0.0</td>
<td>18.2±0.5</td>
<td>18.3±0.5</td>
<td>17.7±0.6</td>
<td>17.4±0.5</td>
</tr>
<tr>
<td>IR64</td>
<td>L</td>
<td>20.9±0.2</td>
<td>19.6±0.8</td>
<td>19.0±0.8</td>
<td>17.8±0.7</td>
<td>17.5±0.7</td>
</tr>
<tr>
<td>IR71</td>
<td>U</td>
<td>20.9±0.2</td>
<td>19.3±0.3</td>
<td>18.7±0.3</td>
<td>17.5±0.2</td>
<td>17.3±0.2</td>
</tr>
<tr>
<td>IR55</td>
<td>L</td>
<td>20.7±0.5</td>
<td>19.8±0.2</td>
<td>18.1±0.2</td>
<td>16.9±0.1</td>
<td>16.6±0.1</td>
</tr>
<tr>
<td>IR71</td>
<td>L</td>
<td>21.2±0.4</td>
<td>20.7±0.5</td>
<td>19.2±0.5</td>
<td>17.7±0.3</td>
<td>17.4±0.3</td>
</tr>
<tr>
<td>IR55</td>
<td>U</td>
<td>21.5±0.3</td>
<td>20.9±0.5</td>
<td>19.3±0.5</td>
<td>17.8±0.4</td>
<td>17.5±0.4</td>
</tr>
<tr>
<td>Van</td>
<td>U</td>
<td>21.1±1.3</td>
<td>18.2±0.5</td>
<td>17.8±0.5</td>
<td>16.5±0.3</td>
<td>16.2±0.3</td>
</tr>
</tbody>
</table>

Values indicate the mean of six plants; indicates the standard error of mean; different letters indicate significant differences (P≤0.05) among means according to an ANOVA.

Yield and A exhibited significant positive correlations with \(g_m\) (Fig. 5a and 5b). However, the positive correlations between yield and A with \(g_m\) (Fig. 5c and 5d) were stronger than those observed with \(g_s\) or total conductance (\(g_{tot}\)), suggesting that \(g_m\) is the major determinant of yield and A under both well-watered and water-stressed conditions. The yield of the O. sativa varieties was strongly related to A under both control and drought treatments (Fig. 5g).

To gauge the response of these diffusive limitations to CO2 transport and their effect on yield and A, the proportional change (\(\Delta\)) in response to drought was calculated. The proportional reduction in yield (\(\Delta yield\)) was not related to \(\Delta g_s\), \(\Delta g_m\) or \(\Delta g_{tot}\) (Fig. 6a; 6b; 6c). However, \(\Delta A\) was significantly correlated to \(\Delta g_m\) and \(\Delta g_{tot}\). IR71 exhibited the highest levels of A and \(g_m\) (Fig. 3a and 3c), but also the lowest proportional reduction in \(g_m\) and A (Fig. 6d). The highest yields observed under control conditions were found in IR55 (3039.6±359.1 kg ha\(^{-1}\)) and IR71 (2407.4±284.4 kg ha\(^{-1}\)) under water-stress the yield of IR55 fell to 52.7±30.3 kg ha\(^{-1}\) and IR71 to 640.3±93.0 kg ha\(^{-1}\). The lowest \(\Delta yield\) reduction under drought conditions was observed in variety Van (Fig. 6), which also produced the highest yield under values of \(A\), \(g_s\) and \(g_m\) under both well-watered and drought conditions, while Apo and PS80 showed the lowest values of these photosynthesis and gas-exchange parameters under control and water-stress. The \(A/g_m\) ratio of the O. sativa varieties either increased (IR71; Van; IR55) or remained constant under drought stress in all varieties with the exception of PS80, where a 42.6% reduction was observed (Fig. 3d). The ratio of [CO2] within the substomatal pore (\(C_i\)) to the external atmospheric [CO2] (\(C_e\)) was largely unaffected by water-stress (Fig. 4a). In contrast, the ratio of the [CO2] within the chloroplast envelope (\(C_s\)) to \(C_e\) was diminished in all O. sativa varieties during drought treatment (Fig. 4b), indicative of transport limitations of CO2 in the mesophyll for photosynthesis. Oryza sativa variety IR71 showed the smallest proportional reduction of 16.8% in the \(C_s/C_e\) ratio, while Apo exhibited the largest reduction of 33.8%.

![Figure 2. Comparison between the estimates of mesophyll conductance of CO2 (g_m) obtained by applying two independent methods: the variable J method and the δ13C of recently synthesised sugars' method (linear regression: \(R^2=0.932\); \(F_{1,6}=122.750\); \(P=1.515 \times 10^{-6}\)). Each data point represents the average value of three observations based upon the \(\Delta 13C\) of recently synthesised sugars and six to fourteen gas-exchange measurements utilizing the variable J method. Error bars as in Figure 1. The regression line excludes the two data points on the right of the graph (IR64 and PS80) with anomalously high \(g_m\) derived from the δ13C of recently synthesised sugars. Numbers next to data points indicate Oryza sativa variety as in Figure 1. doi:10.1371/journal.pone.0109054.g002](image-url)
IR71 exhibited the second lowest yield and produced second largest yield during water-stress of the varieties analysed (Fig. 6g). The A/gs ratio was not significantly related to either yield or HI under both well-watered or drought conditions (Fig. 7a and 7c). However, the gms/gs ratio was significantly correlated with both yield and HI (Fig. 7b and 7d), suggesting that those O. sativa varieties with greater gms/gs ratio will exhibit enhanced productivity under both well-watered and water-stressed conditions.

Discussion

The seven O. sativa varieties exhibited a diverse range of morphological, isotopic, photosynthetic and leaf gas-exchange responses to drought stress. A number of factors determined the yield of the O. sativa cultivars analysed in this study. Despite water deficit being instigated at the same developmental stage for all varieties, phenology had an effect on yield under drought as rapidly developing plants maintained yield to a greater extent than slower developers (Fig. 1a) [52]. Despite the type and phenological timing of the drought stress being different to that utilised in the investigation of Centritto et al. [7], the effect on A and yield observed in the two studies are broadly consistent. Those plants that were able to accumulate biomass rapidly produced higher...
Figure 5. Interaction of diffusive conductance parameters to CO₂ uptake with yield and photosynthesis (A) under well-watered (open symbols) and drought conditions (closed symbols): a) relationship between yield and stomatal conductance (gs) (linear regression: $R^2 = 0.696$; $F_{1,10} = 22.900$; $P = 0.000740$); b) relationship between A and gs (linear regression: $R^2 = 0.873$; $F_{1,11} = 75.721$; $P = 2.911 \times 10^{-6}$); c) relationship between yield and mesophyll conductance (gm) (linear regression: $R^2 = 0.850$; $F_{1,10} = 56.611$; $P = 2.911 \times 10^{-5}$); d) relationship between A and gm (linear regression: $R^2 = 0.952$; $F_{1,11} = 217.071$; $P = 1.376 \times 10^{-8}$); e) relationship between yield and total conductance (gtot) (linear regression: $R^2 = 0.806$; $F_{1,10} = 41.418$; $P = 7.483 \times 10^{-5}$); f) relationship between A and gtot (linear regression: $R^2 = 0.869$; $F_{1,11} = 1.407 \times 10^{-8}$); and; g) relationship between yield and A (linear regression: $R^2 = 0.795$; $F_{1,11} = 38.665$; $P = 9.909 \times 10^{-5}$). Error bars as in Figure 1. Numbers next to data points indicate Oryza sativa variety as in Figure 1. doi:10.1371/journal.pone.0109054.g005
Figure 6. Changes in yield and photosynthesis in relation to modification of diffusive resistances to CO₂ uptake following water-stress. Those varieties that experienced smaller reductions in parameters were more tolerant of drought. a) relationship between Δyield and Δgₛ (linear regression: $R^2 = 0.337$; $F_{1,4} = 2.032$; $P = 0.227$); b) relationship between Δyield and Δgₘ (linear regression: $R^2 = 0.134$; $F_{1,4} = 0.618$; $P = 0.476$); c) relationship between Δyield and Δgₜₜ (linear regression: $R^2 = 0.0818$; $F_{1,4} = 0.356$; $P = 0.583$); d) relationship between ΔA and Δgₛ (linear regression: $R^2 = 0.0003$; $F_{1,4} = 0.00106$; $P = 0.976$); e) relationship between ΔA and Δgₘ (linear regression: $R^2 = 0.742$; $F_{1,4} = 11.527$; $P = 0.0274$); f) relationship between ΔA and Δgₜₜ (linear regression: $R^2 = 0.715$; $F_{1,4} = 10.042$; $P = 0.0339$); and; g) relationship between Δyield and ΔA (linear regression: $R^2 = 0.427$; $F_{1,4} = 2.979$; $P = 0.159$). Error bars as in Figure 1. Numbers next to data points indicate Oryza sativa variety as in Figure 1.

doi:10.1371/journal.pone.0109054.g006
grain yields than those that accumulated biomass less efficiently under both well-watered and drought conditions. This may be due to allometric relationships between biomass and yield [53], or the positive influence of increased photosynthetic area on ‘whole-plant’ carbon uptake [54]. Leaf-level photosynthetic rates were also closely related to yield in all varieties under both well-watered and drought conditions (Fig. 5g). Photosynthesis rates were in turn determined by diffusive limitations to CO2-uptake (Fig. 5b; 5d; 5f). The most significant of these resistances to CO2-uptake in *O. sativa* occurs at the interface between the internal sub-stomatal air-space and the mesophyll layer [15,55]. Consistent with other studies, gm in *O. sativa* correlated most closely with A and yield (Fig. 5d) [7,9], and represents a critical ‘rate-limiting’ step in the determination of grain productivity and assessment of the photosynthetic response to drought stress.

The ‘bulk’ carbon isotope discrimination values of the *O. sativa* varieties decreased following drought treatment; indicative of water-deficits over the life-span of the flag-leaf reducing discrimination against the heavier 13C isotope [56,57]. However, this pattern of reduced carbon isotope discrimination is not apparent in the carbon isotopic composition of recently synthesised sugars (Table 1). These findings may reflect ‘shorter-term’ variations in WUE in the plants experiencing drought stress, a modification of the photosynthetic pathway affecting sugar metabolism [28] or the breakdown of previously stored carbohydrates with a different isotopic signature into more simple sugars over the course of the stress treatment [58,59]. Differences in the diffusional uptake of CO2 likely affected carbon isotopic composition [60]; however, this was not apparent from our gm, gs, variable J datasets (Fig. 5), suggesting that other factors affected carbon isotopic discrimination and possibly the effectiveness of the Δ13C of recently synthesised sugars method of estimating gm in varieties IR64 and PS80 under control conditions (Fig. 2). It is noteworthy that under water-deficit conditions, gm estimates based on the variable J method closely correlated with those derived from the Δ13C of recently synthesised sugars (Fig. 2). These findings do not support the suggestion that reductions in Ci may affect estimation of the total photosynthetic electron transport rate associated with enhanced photorespiration, thus rendering the variable J method unreliable in the investigation of gm in plant responses to environmental stress [34].

Selective breeding has improved the productivity of *O. sativa* varieties through improved growth form and the increased proportional allocation of biomass as grain [4]; yet these increases in yield have been constrained by the relatively low leaf-level photosynthetic rate of *O. sativa* in comparison to other C3 crops [5,61]. The results of this study indicate that under well-watered and water-deficit conditions, A is an important determinant of yield in *O. sativa* and is largely related to the rate of transport of CO2 across the mesophyll (Fig. 5d). Further improvements in *O. sativa* grain yield may likely be achieved through the enhancement of gm to CO2 inducing enhanced A [6,9,16,62]. Those *O. sativa* varieties that showed the greatest photosynthetic performance and yield under control conditions were not necessarily the most
effective varieties under drought conditions. Varieties that maintained transport of CO$_2$ during water-stress sustained $A$ and yield to a greater extent than varieties that exhibited more pronounced diffusive limitations to photosynthetic CO$_2$-uptake (Fig. 5). Drought stress induced a relatively consistent mean reduction in $g_m$ in the *O. sativa* varieties (Fig. 3), yet the $C_i/C_a$ ratio was unaffected (Fig. 4), indicating that $g_s$ was not limiting to $A$ under water-deficit, or that the gas-exchange data were possibly biased by the occurrence of non-uniform stomatal closure over the leaf lamina and consequent overestimation of $C_i$ [27,63,64]. However, the $C_i/C_a$ ratio in the *O. sativa* varieties was reduced by 16.8 to 33.8% after drought, consistent with the impairment of $A$ through mesophyll diffusive limitations [7,11,12,65,66]. Mesophyll conductance to CO$_2$ declined during the drought treatment, constraining $A$ and growth (Fig. 5; 6). However, significant variability in the extent of reductions in $g_m$ were observed in the *O. sativa* cultivars ranging from −61.7% (IR71) to −89.3% (PS80). Furthermore, those varieties that showed the smallest reductions in $g_m$ also exhibited greater $A$; demonstrating that $g_m$ largely determines $A$ in *O. sativa* under differing degrees of water-deficit [7,9]. These differences in the ability to sustain $g_m$ values under drought stress are likely related to the maintenance of biochemical function in the mesophyll such as the activity of cooparin and carbonic anhydrase [eg. 25,67].

The conductance of the mesophyll layer to the transport of CO$_2$ determined $A$ and yield in the seven *O. sativa* varieties studied. Under conditions of water-stress it has been suggested that a low $g_s$ (to reduce transpirative water-loss) and a high $g_m$ (to maintain the uptake of CO$_2$) are beneficial [11,68]. However, in the case of *O. sativa*, yield and HI were only weakly related to the ratio of $g_m/g_s$, ($R^2<0.460$) (Fig. 7). The reduction in $g_s$ under water-deficit is consistent with active stomatal control in the optimisation of WUE [69,70]; nevertheless, the lack of a strong correlation to the $g_m/g_s$ ratio may be indicative of the lack of a stomatal limitation to $A$ under drought stress in the *O. sativa* varieties apparent in the $C_i/C_a$ ratios (Fig. 4a). The photosynthetic and yield responses of *O. sativa* under well-watered and drought conditions were more dependent upon $g_m$ than $g_s$ (Fig. 5b and 5d). This is consistent with earlier studies reporting that modification and improvement of $g_m$ is more effective in the enhancement of *O. sativa* productivity [16] and likely drought tolerance [7,9]. The strong dependence of $A$ and yield on $g_m$ is also evident in the lack of a relationship between $A/g_s$ and yield or HI (Fig. 7a and 7c). This may suggest that stomatal control is either less effective [70,71], or plays a reduced role, in respect to mesophyll limitations to CO$_2$ in a drought intolerant crop such as *O. sativa* in comparison to crops adapted to more water-limited environments [72].

The disparity in $A$ values observed between the cultivars raises the possibility of crossing different varieties to enhance leaf-level $A$ and yield in subsequent generations [6,8,16]. Selection of drought tolerance on the basis of the ability of a particular genotype to maintain $g_m$ function under drought stress would be viable [11,73]. However, genetic sequencing and identification of quantitative trait loci permit the possibility of extracting genetic material from a *O. sativa* cultivar with a valuable characteristic (such as high $g_m$ and/or stable $g_m$ under water deficit) and in the creation of a new variety with a suite of desired attributes [17,19,74–76]. For example, from the results of this study it would be desirable to combine the high $g_m$ and yield of IR55 under well-watered conditions with the capacity to sustain $g_m$ under water-stress exhibited by IR71 and water-stressed yield of Van (Fig. 3). In this context it may be possible to phenotypically screen a large number of *O. sativa* genotypes to identify quantitative trait loci responsible for $g_m$ values and $g_m$ response to drought. Those varieties developed to be resistant to drought may be suited to more marginal land-types or in scenarios of insufficient water availability. Nonetheless, given the sporadic and unpredictable nature of sustained and persistent drought events it is unlikely that large numbers of farmers would select a drought tolerant variety such as Van over a more productive cultivar such as IR55, and thus limit their harvest to accommodate the risk of interruption to water supplies [73,77]. An ideal *O. sativa* variety would therefore possess both values of $g_m$ capable of sustaining high $A$ and the ability to maintain $g_m$ in the event of water-deficits [8,78]. However, the results of this study suggest that those attributes may be mutually exclusive in the species analysed. Furthermore, no single parameter analysed in this study exclusively accounted for yield under either well-watered or drought conditions; therefore attempts to improve the productivity of *O. sativa* cultivars may also involve selection for a suite of characteristics that improve productivity under specific conditions of water availability. Nonetheless, future efforts to improve *O. sativa* productivity should focus on biochemical [eg. 25] and physical modification [eg. 16] of the interface between the mesophyll layer and sub-stomatal air-space to enhance CO$_2$ uptake and $A$.

**Conclusions**

Physiological analysis of *O. sativa* has great potential in identifying those photosynthetic and leaf gas-exchange characteristics that confer attributes such as high yield and tolerance to drought. The analysis of the seven *O. sativa* varieties indicates that under both well-watered and drought stressed conditions yield and $A$ are determined by diffusional constraints to CO$_2$ uptake and not by biochemical limitations. The varieties showed significant variation in leaf-level photosynthetic rates, symptomatic of the photosynthetic constraints to *O. sativa* production that have previously been recorded. Selection of those *O. sativa* genotypes that exhibit higher rates of leaf-level $A$ would likely be beneficial for grain yields. Photosynthesis was most closely related to $g_m$ and the tolerance of the *O. sativa* varieties to drought was largely determined by the ability to maintain $g_m$ values under water-deficits. However, the highest $g_m$ and yield under well-watered conditions (IR55) and the most pronounced ability to sustain $g_m$ under water-deficit (IR71) were observed in different varieties. This may suggest that the desired attributes for enhanced yield during high water availability and the ability to maintain $g_m$ and thus sustain grain production under water-deficits, may be mutually exclusive. Nonetheless, the results of studies such as this could make it possible through selective breeding, or the identification of a particular genetic expression associated with a desired attribute, to increase leaf-level photosynthetic rates and drought tolerance in the same variety of *O. sativa*. These findings indicate that the interface between the internal sub-stomatal air-space and the mesophyll layer offers the greatest potential in terms of possible modification to enhance the performance of *O. sativa* under optimal and stressed growth conditions in the future.

**Supporting Information**

**Figure S1** Dark respiration ($R_d$) values of seven *Oryza sativa* varieties under irrigated (white) and rain-fed (grey) conditions used in the calculation of $g_m$ using the variable J method. (PDF)

**Figure S2** Electron transport rate (ETR) values of seven *Oryza sativa* varieties under irrigated (white) and rain-
fed (gray) conditions used in the calculation of $g_m$ using the variable J method. (PDF)

Acknowledgments

The following are thanked for scientific discussion and technical assistance: L. Spaccino for IRMS analyses (CNR-IBAF) and R. Torres G. Dimayuga and the drought physiology team at IRRI for technical support (IRRI, Philippines). The comments of two anonymous referees significantly improved this manuscript.

Author Contributions

Conceived and designed the experiments: MC ML RS. Performed the experiments: MC ML MCM. Analyzed the data: MH MC ML. Contributed reagents/materials/analysis tools: MC ML RS. Wrote the paper: MH MC ML.

References