**UNIVERSIDADE ESTADUAL PAULISTA**
**"JÚLIO DE MESQUITA FILHO"**
**INSTITUTO DE BIOCIÊNCIAS – RIO CLARO**

**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS**
**(BIOLOGIA VEGETAL)**

**VERTICALIDADE FOLIAR E ASPECTOS FOTOSSINTÉTICOS**

**REVISITADOS**

**EDUARDO BORGES DA VEIGA**

Tese apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Biologia Vegetal).

**Março - 2016**

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A Deus

"Adquirir a sabedoria vale mais que o ouro; antes adquirir a inteligência que a prata (Pro 16, 16)".

A minha família

Meu pai Zeferino, minha mãe Bárbara, minha irmã Roniela e meu sobrinho Pedro Luiz.

A Diane Matias C. Silva

Pessoa mais que especial.

**DEDICO**

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# SUMÁRIO

**RESUMO**

Aqui analisamos o papel ecofisiológico da verticalidade foliar na eficiência de uso da luz pelas plantas. Os estudos foram conduzidos em espécies de *Styrax camporum* e *Saccharum* spp. A verticalidade foliar tem papéis distintos entre as diferentes espécies, mas primordialmente tais resultados convergem para um melhor aproveitamento da luz pela planta com folhas dispostas nessa conformação, com ganho de biomassa.

**ABSTRACT**

Here we analyzed the ecophysiological role of vertical leaves in sunlight use efficiency by plants. The studies were conducted in species of *Styrax camporum* and *Saccharum* spp. Vertical leaves have distinct roles between different species, but primarily these results demonstrate that plants with vertical leaves show a better light use efficiency, eventually with gain of biomass.

**INTRODUÇÃO**

A luz solar é uma das fontes de energia mais importantes na Terra e sua captação e processamento por seres vivos podem significar a permanência de uma espécie na biota terrestre (Lawlor 2001a). As folhas das plantas são os órgãos especializados na captação e processamento da luz (Lawlor 2001a). Tal aproveitamento da luz é, industrialmente, ainda muito pouco utilizado, muito caro, pouco eficiente e até inviável.

Nesse sentido, o uso de energias alternativas vem crescendo mundialmente. Dentre os usos alternativos da energia está a tecnologia de painéis fotovoltaicos. Porém, um dos principais problemas dos painéis é que ainda não foi encontrada uma maneira eficiente e barata de converter a energia fotovoltaica em energia química, imitando a fotossíntese (Ereno & Oliveira 2011). Ainda assim, estudos recentes têm mostrado que a melhor forma de otimizar a eficiência desses painéis seria dispô-los à maneira do arranjo das folhas em uma planta (Lourenço 2011). As plantas, por outro lado, como já têm a fotossíntese estabelecida em suas folhas (embora também com baixa eficiência – Melis 2009), vivem outros "dilemas". No ambiente natural, onde evoluíram, enfrentaram e ainda enfrentam pressões ambientais que vão desde hábitats áridos com alta disponibilidade de luz, até ambientes florestais sombreados e geralmente úmidos, passando ainda por ambientes sazonais: estações frias e verões quentes, por exemplo (Raven et al. 2001).

As "estratégias" das plantas de ambientes áridos e com muita luz parecem se agrupar. Muitas das espécies de deserto são plantas suculentas que desenvolveram um metabolismo envolvendo ácidos orgânicos (málico, aspártico etc) e os poros estomáticos abrem-se somente no período noturno (Lawlor 2001b). Outro hábitat sujeito à alta carga de irradiância, geralmente acompanhado da estação seca, são as savanas (Oliveira &

Marquis 2002, Ferreira et al. 2003, Luttge 2008). Nas savanas, sobretudo na brasileira (Cerrado), as estratégias mais recorrentes são o sistema radicular profundo (Franco 1998, Habermann & Bressan 2011), folhas coriáceas, com baixos valores de área foliar específica (AFE), folhas com orientação vertical (Caldas et al. 1997, Habermann et al. 2008), forte ajuste osmótico, dentre outras.

Assim, parece que as diferentes pressões ambientais selecionaram plantas com características específicas para sobreviver com recursos limitantes ou superar efeitos negativos causados por recursos em excesso (Ackerly et al. 2000). Um dos recursos considerados em excesso para as plantas é a luz, uma vez que cerca de 80% da luz interceptada pelo aparato fotossintético seria desperdiçada (Melis 2009).

Assim, a forma de disposição das folhas numa planta assume grande importância. De fato, em gradientes ambientais, parece haver alguma relação entre o aumento da aridez e o tamanho da folha e altura da planta, que diminuem, ficando as folhas mais espessas e em posições cada vez mais verticais (Ehleringer 1988, Smith et al. 1998). Portanto, a imediata e intuitiva associação entre o posicionamento vertical das folhas e a "estratégia" para evitar o excesso de luz, estabelece-se. Essa hipótese ganha força com o fenômeno do paraheliotropismo, que é o movimento foliar em orientação paralela ao eixo principal dos raios solares, que seria oposto ao diaheliotropismo, ou seja, folhas interceptando perpendicularmente os raios solares, o que lhes garantiu a denominação científica de "*solar tracking plants/leaves*" (Sailaja & Rama Das 1996, Takahashi & Badger 2011). *Erythrina speciosa* (Fabaceae) é uma leguminosa nativa que apresenta típico comportamento foliar paraheliotrópico. Em algumas espécies, folhas horizontais podem também ser chamadas de folhas diaheliotrópicas sem, no entanto acompanharem perpendicularmente a posição do sol (Habermann et al. 2008). Contudo, estas seriam melhor denominadas folhas estáticas (Feistler & Habermann 2012). Parte dos relatos de

para- ou diaheliotropismo vêm dos estudos com espécies leguminosas nativas (Rodrigues & Machado 2006, Arena et al. 2008), mas sobretudo espécies cultivadas (Pastenes et al. 2005, Jiang et al. 2006, Zhang et al. 2011).

Os movimentos paraheliotrópicos podem ser causados por diversas condições ambientais, tais como déficit hídrico (Pastenes et al. 2005, Zhang et al. 2011), aquecimento foliar e excesso de irradiância (Caldas et al. 1997, Bielenberg et al. 2003, Jiang et al. 2006). A menor interceptação de luz e reduzido superaquecimento foliar, causado pelo movimento paraheliotrópico, faz com que o desempenho fotoquímico, evidenciado pela eficiência quântica potencial ($F_v/F_m$) e efetiva ($\Phi_{PSII}$) do fotossistema II (PSII) e taxa aparente de transporte de elétrons (ETR) de folhas paraheliotrópicas seja maior que folhas diaheliotrópicas (Arena et al. 2008). Logo, a fotoproteção paraheliotrópica parece ser uma hipótese bem aceita (Takahashi & Badger 2011).

Contudo, algumas variações dos efeitos do paraheliotropismo já foram relatadas. Alguns estudos (Arena et al. 2008) mostram que as folhas paraheliotrópicas evitam o excesso de luz, diminuem a temperatura da folha e a transpiração foliar (*E*), melhorando a eficiência fotoquímica (evitando fotoinibição) e, por causa disso, aumentam a condutância estomática (*gs*) e a assimilação de $CO_2$ (*A*). Outros estudos em feijoeiro (Pastenes et al. 2005) mostram que o paraheliotropismo como resposta protetora contra a deficiência hídrica, mesmo protegendo o aparato fotossintético, não aumenta ou sequer mantém a assimilação de carbono, que encontra-se sob forte controle de *gs*, significativamente reduzida pela deficiência hídrica. Para feijoeiros irrigados, Pastenes et al. (2004 e 2005) observaram que embora o paraheliotropismo evite o aquecimento foliar e melhore a eficiência fotoquímica da folha, ao evitar a interceptação de luz, inibe ganhos potenciais de carbono. Corroborando parte dos resultados de Pastenes et al. (2005), Bielenberg et al. (2003) observaram que o paraheliotropismo em feijoeiro protege a planta

como um todo porque ocorre aumento da eficiência de uso da água ($A/E$). Estas mesmas conclusões também já tinham sido apontadas para a soja (Kao & Tsai 1998).

Mesmo espécies não leguminosas, como o algodoeiro, mostram que o posicionamento vertical induzido pela deficiência hídrica (folhas caídas, mas não paraheliotrópicas) pode ser uma "estratégia" de proteção contra o excesso de irradiância combinado à falta de água no mesofilo (Zhang et al. 2010). Contudo, comparações feitas entre plantas de algodão e soja sob deficiência hídrica mostram que as respostas são diferentes. Enquanto a soja responde com movimento paraheliotrópico à deficiência hídrica, as folhas de algodoeiro mantém o diaheliotropismo até o ponto de murcha; além disso, plantas de soja sob deficiência hídrica apoiam sua proteção no aumento de processos não assimilativos, com elevada taxa de extinção não fotoquímica da fluorescência (NPQ) e transporte eletrônico cíclico, ao passo que as folhas de algodoeiro aumentam ETR, evidenciando o aproveitamento da luz e assimilação de nitrogênio (Zhang et al. 2011). Logo, apesar das variações observadas para diferentes espécies e sob diferentes circunstâncias, o paraheliotropismo descrito em diversas famílias de plantas tem um papel fotoprotetor importante (Takahashi & Badger 2011).

Existem ainda espécies que apresentam folhas verticais estáticas (Falster & Westoby 2003, Liu et al. 2003, Feistler & Habermann 2012), que além de proporcionar menor interceptação de luz, também podem envolver duas outras hipóteses relacionadas ao aproveitamento da luz. Folhas verticais estáticas podem manter altos valores de $A$ durante períodos de alta irradiância ao meio dia mas também podem proporcionar boa interceptação diária de radiação do sol em ângulos menores no céu, como de manhã, à tarde, no inverno e em locais de alta latitude (Falster & Westoby 2003), incutindo uma ideia de melhor eficiência diária de uso da luz. No entanto, estimativas indiretas desses autores indicaram que o fator proteção parece ser mais importante.

*Styrax camporum*, um arbusto do Cerrado, possui folhas verticais e folhas horizontais estáticas (Feistler & Habermann 2012). Contudo, as folhas verticais dessa espécie mostram maiores valores de *A*, *E* e *gs* em relação às folhas horizontais (Habermann et al. 2008, 2011). Além disso, a temperatura das folhas verticais não é menor que a das folhas horizontais (Habermann et al. 2008, Feistler & Habermann 2012). Ainda, as folhas verticais dessa espécie não mostram maior eficiência fotoquímica em relação às folhas horizontais de plantas irrigadas ou sob déficit hídrico (Feistler & Habermann 2012). Em relação ao conceito de fotoproteção, as folhas verticais de *S. camporum* são diferentes de muitos relatos da literatura. Logo, duas ideias estabelecem-se com esses resultados nessa espécie: que as folhas verticais promovem maior eficiência de uso da luz (Falster & Westoby 2003, Habermann et al. 2008, 2011) e que as folhas verticais de *S. camporum* podem promover maior penetração de luz na copa (Feistler & Habermann 2012).

A ideia de maior eficiência de uso da luz pelas folhas implica na interceptação de luz por ambas as faces foliares ao longo do dia. De fato, curvas de assimilação de $CO_2$ em função da variação do fluxo de fótons fotossinteticamente ativos (*A*/FFFA) indicam que a capacidade fotossintética das folhas verticais de *S. camporum* é similar entre suas faces, enquanto esta mesma capacidade é distinta entre as faces das folhas horizontais (Habermann et al. 2011). Apesar dessas consideráveis capacidades fotossintéticas das faces foliares, a face abaxial de folhas de *S. camporum* possui maior refletância no espectro fotossintético (400 – 700 nm), comparada à face adaxial (Habermann et al. 2011), propriedade ótica da folha esta que aumenta a fluorescência observada na face abaxial (Feistler & Habermann 2012) dessa espécie. Esses resultados corroboram observações para outras espécies (Cordón & Lagorio 2007). Há ainda relatos demonstrando que folhas igualmente iluminadas em ambas as faces exibem maiores

valores de *A* do que quando iluminadas apenas em uma das faces (Proietti & Palliotti 1997).

Por outro lado, o diaheliotropismo foi defendido (Ehleringer & Forseth 1980) como uma forma de aumentar a capacidade produtiva de espécies cultivadas, espelhando-se em espécies nativas de deserto. Porém, mais estudos em plantas nativas eram sugeridos àquela época (Ehleringer & Forseth 1980) como potenciais substratos de conhecimento para o benefício de plantas cultivadas. Propôs-se ainda que os mecanismos de (foto) proteção em plantas para- e diaheliotrópicas são distintos para o aumento da performance fotossintética (Sailaja & Rama Das 1996).

Até agora, com algumas variações e correções, todos os modelos fazem sentido. Como descrito anteriormente, o (para- e dia-) heliotropismo e a verticalidade foliar causam efeitos diversos (Pastenes et al. 2005, Arena et al. 2008, Habermann et al. 2008, Zhang et al. 2011, Feistler & Habermann 2012), com diferentes possibilidades interpretativas, embora a idéia de fotoproteção seja quase universal. Portanto, aparentemente, as diferentes respostas devem estar ligadas às diferentes capacidades fotossintéticas máximas da copa e da folha e estas respostas parecem ser dependentes da diversidade de espécies (Pastenes et al. 2005, Zhang et al. 2011), ou, generalizando-se, pode-se até dizer que a questão é ainda "mal resolvida". Isso porque embora possa parecer que folhas diaheliotrópicas ou horizontais estáticas interceptem mais luz e possam se beneficiar disso com ganhos de carbono, também é forte a ideia da fotoproteção de folhas paraheliotrópicas ou verticais.

Também é bem aceito que folhas verticais possam se beneficiar de baixos ângulos solares de manhã e à tarde, assim como interceptação de luz por ambas as faces ao meio dia. A maior performance fotossintética em folhas diaheliotrópicas ou *solar tracking leaves* poderia ser explicada pela característica de constância na eficiência do PSII mesmo

sob altas cargas de irradiância (Sailaja & Rama Das 1996). Ainda, modelos computacionais sugerem que os efeitos de folhas do tipo *solar tracking* podem ser negativos sobre a produtividade da comunidade de plantas (Denison et al. 2010).

Sem negligenciar a importante e bem aceita hipótese de fotoproteção (Takahashi & Badger 2011), resta revisar os métodos de medição das variáveis mais estudadas nesse contexto. A maioria dos estudos envolvendo verticalidade ou horizontalidade foliar por heliotropismo e/ou folhas estáticas normalmente consideram variáveis de trocas gasosas medidas em apenas uma das faces foliares, geralmente a face adaxial e, com iluminação artificial. Medidas feitas com o medidor de trocas gasosas LI-6400 (LI-COR, Lincoln-NE, EUA), usando luz artificial de LED (6400-02B, LI-COR, EUA) na face adaxial de plantas de soja e algodoeiro foram recentemente relatadas (Zhang et al. 2010, Zhang et al. 2011).

Os mesmos métodos gerais (medição com iluminação artificial apenas na face adaxial) foram relatados, com o medidor CIRAS-I (PPSystem, RU), com controle da temperatura e do $CO_2$ na câmara (Pastenes et al. 2004, Jiang et al. 2006). Pastenes et al. (2005) utilizaram o medidor ADC-pro (ADC Bioscientific Ltd, RU), que se utiliza de iluminação natural ou artificial na face adaxial. Bielenberg et al. (2003) fizeram medidas com o medidor de trocas gasosas LI-6252 (LI-COR, Lincoln-NE, EUA), com luz artificial em uma das faces foliares, provavelmente a face adaxial. Arena et al. (2008) fizeram medidas de trocas gasosas em folhas de *Robinia pseudoacacia*, que apresenta movimento paraheliotrópico, utilizando o medidor HCM-1000 (Walz, Effeltrich, Alemanha) com luz natural incidente na face adaxial.

Há poucos estudos em que se tenham utilizado câmaras transparentes. Uma câmara transparente pode permitir a interceptação de luz direta por uma das faces foliares e a interceptação de luz difusa pela outra face. Liu et al. (2003) e Habermann et al. (2008)

utilizaram o medidor LI-6200 (LI-COR, Lincoln-NE, EUA) com câmara transparente, mas encontraram resultados opostos, sobretudo com relação à hipótese do papel foto- e termo- protetor das folhas verticais, embora tenham trabalhado com espécies diferentes. Já os parâmetros de fluorescência da clorofila *a* são usualmente e largamente medidos na face adaxial das folhas, embora seja possível medir tais parâmetros em ambas as faces (mas não ao mesmo tempo), como realizado por Feistler & Habermann (2012).

Pode-se argumentar que as folhas de um grande número de espécies são hipo-estomáticas, pilosas e com maior refletância na face abaxial. *S. camporum* apresenta todas essas características e embora em folhas verticais os valores de *A* sejam menores na face abaxial sob FFFA não saturante (até 800 µmol fótons $m^{-2}$ $s^{-1}$), acima dessa intensidade de luz as capacidades fotossintéticas de ambas as faces igualam-se (Habermann et al. 2011). Os equipamentos medidores de trocas gasosas foram supostamente desenvolvidos para medidas com iluminação (usualmente artificial) na face adaxial das folhas, porém as marcas oferecem câmaras para diferentes condições.

Logo, ao medir a assimilação de $CO_2$ foliar sem associar com a produtividade biológica e/ou agronômica da planta, ou ao medi-la apenas na face adaxial, pode-se subestimar a capacidade fotossintética de toda a planta, em um estado momentâneo, ou ao longo do dia. É também válido destacar que características momentâneas, como as respostas de trocas gasosas mostram baixa herdabilidade, ao passo que características morfológicas, como o posicionamento foliar, podem ser mais substancialmente herdáveis (Ackerly et al. 2000). Assim, a penetração de luz na copa de uma planta, o papel fotoprotetor da verticalidade foliar e a maior eficiência de uso da luz pelas folhas são características que poderiam melhor orientar programas de melhoramento genético de diferentes espécies como uma alternativa ao aumento da captação de luz que é grandemente desperdiçada (Melis 2009), refletindo-se em maior produção de biomassa.

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**CHAPTER 1**

**CO$_2$ assimilation rates as measured with different leaf chambers: the use of a full clear chamber to measure static vertical leaves of *Styrax camporum***

**Abstract**

*Styrax camporum* is a plant from the Cerrado often observed at the edge of vegetation fragments and has horizontal and vertical static leaves. We analyzed gas exchange rates of adult plants grown in pots. Measurements were performed with leaf chambers that allow the natural passage of sunlight (*Combination Chamber* and *Full Clear Chamber*) and also with a chamber that possesses artificial light (LED *source*) illuminating one of the leaf sides. We analyzed the adaxial and abaxial sides of leaves showing different (petiole) angles in relation to the horizon. The LED *source* chamber showed great uniformity in the results throughout the day, and when iluminating the abaxial side values were similar to those of the adaxial one. In vertical leaves, the *Combination Chamber* returned lower carbon assimilation in relation to the other two chambers, even when measuring the adaxial and abaxial sides. When using the *Full Clear Chamber* and allowing illumination of the abaxial side, it returned high gas exchange values at the early hours of the day, similar to the results obtained with LED *source*. There was no relationship between leaf angles and leaf temperature. Both leaf types showed different gas exchange rates throughout the day, but such different angles may have an important ecophysiological role to light usage by the whole plant. In addition, we demonstrate the importance of the abaxial leaf side to gas exchange measurements.

**Keywords:** gas exchange, leaf angles, leaf chambers, Styracaceae.

**Introduction**

During a plant's lifecycle, it is subject to a number of environmental factors, such as daily and seasonal influences. These factors can affect physiological processes essential to plant growth. Leaves can respond to these factors showing momentaneous variations in gas exchange, which can distinguish species from different successional status (Ribeiro *et al.* 2004), identify different strategies for plants inhabiting specific environments (Kao and Forseth 1991, Possel and Hewitt 2009, Zhang *et al.* 2011), and also evaluate the photochemical performance when associated with fluorescence measurements (Zhang *et al.* 2009, Zhang *et al.* 2010). However, these measurements are, in general, conducted on the adaxial leaf surface using a specific leaf area.

There are some possibilities for analyzing gas exchange, depending on the aim of the study. However, different leaf angles may represent an unusual concern for gas exchange (Liu *et al.* 2003). As leaf orientation can determine the proportion of irradiance intercepted by a leaf, especially in environments with high irradiance loads, the procedures for measuring gas exchange can also affect the results of carbon assimilation throughout the day. In addition, the canopy architecture can enhance productivity (Long *et al.* 2006) and the angle of leaf insertion is critical when considering the plant architecture.

Leguminous species can move the leaf lamina so as to intercept sunlight in a perpendicular (diaheliotropism) or parallel (paraheliotropism) conformation to the direct sun rays. These movements are especially pronounced when these species are exposed to high irradiance (Jiang *et al.* 2006), high temperature (Raeini-Sarjaz and Chalavi 2008) and water deficit (Kao and Forseth 1991, Pastenes *et al.* 2004a, Xu *et al.* 2009). These studies have revealed the importance of paraheliotropism in photoprotection, either when associated or not with carbon gains. Alternatively there are plants exhibiting static

horizontal and vertical leaves, such as in *Styrax camporum* (Habermann *et al.* 2008). In this species, leaves with larger angles relative to the horizontal plane (similar to paraheliotropic) show greater photosynthetic gain, denoting a higher importance than the photoprotective role (Habermann *et al.* 2011). The increased importance of carbon gain in detriment to the photoprotective role was also found in *Gossypium hirsutum*, although in horizontal leaves (Zhang *et al.* 2009). Therefore, the study of different leaf conformations enables not only the understanding of ecophysiological significance of this phenomenom, but also the use of these results in plant breeding programs.

There are a wide variety of devices and leaf chambers used in gas exchange measurements. However, most measurements are made with chambers with artificial light in the red and blue bands allowing illumination of only one leaf side, and usually the adaxial side is assessed (Zhang *et al.* 2009, Zhang *et al.* 2010, Habermann *et al.* 2011). Perhaps, the leaf conformation and leaf side should be considered mainly when it involves the study of sunlight usage and/or daily measurements (Feistler and Habermann 2012).

We investigated some sets of leaf chambers of the LI-6400*xt* (LI-COR, USA), with light interception by only the adaxial, only the abaxial, and by both leaf sides, using leaf chambers that allow the natural passage of sunlight on the top and at the bottom of the chamber, as well as a chamber with artificial light that illuminates only one of the leaf sides. Measurements were assessed in static vertical and horizontal leaves of *S. camporum* plants. We predicted that (i) the different leaf chambers return distinct values of $CO_2$ assimilation (*A*), (ii) adaxial and abaxial leaf sides show similar values of *A* when measured with the chamber possessing artificial light, (iii) chambers allowing illumination by both leaf sides enhance the leaf's $CO_2$ assimilation capacity, and (iv) vertical and horizontal leaves show distinct patterns of *A* throughout the day.

**Material and methods**

**Plant Material**

We used five three-year-old plants of *Styrax camporum* Pohl. with 1.0 m in height planted in 100 L pots in a experimental garden at the São Paulo State University, Rio Claro (22º 23' 47.06" S, 47º 32' 39.87" W), São Paulo State, Brazil. These plants exhibited vertical and horizontal static leaves.

**Experimental description and leaf chambers**

We measured gas exchange rates on mature fully expanded vertical and horizontal leaves, using a portable open-gas infrared analyzer, LI-6400xt (LI-COR, Lincoln, NE, USA). We managed the leaf chambers in such a manner to enable light interception by only the adaxial, only the abaxial, and by both leaf sides.

One of the leaf chambers was the 6400-02B (LI-COR, USA), which enabled artificial red (90%) and blue (10%) LED light interception by only one leaf side. This light source was held on the opaque top of the 6 $cm^2$ (2 x 3 cm) leaf chamber. This leaf chamber will be named here 'LED *source*'.

We also used a combination of LI-COR chambers in which the *Sun + Sky Chamber* (LI-COR, USA) was set up on top of the 6 $cm^2$ leaf chamber and the *Clear Bottom Chamber* (LI-COR, USA) at the bottom of it, allowing natural irradiance through a propafilm® window (on a metal frame) to be intercepted by both leaf sides. Therefore, this leaf chamber will be named here '*Combination Chamber*'. In this way, we also manipulated this *Combination Chamber*, so that when covering up (with a piece of cardboard) the top of this chamber, natural light was intercepted only by the abaxial leaf side, and when covering up the bottom of this chamber, sunlight was intercepted only by the adaxial leaf side.

In addition, we made a third leaf chamber using plexiglass that will be named here '*Full Clear Chamber*'. This chamber was similar to the *Combination Chamber* (6 cm$^2$), but while metal is used for the *Combination Chamber* frame (2 x 3 cm), the *Full Clear Chamber* is entirely made of plexiglass, being totally transparent (Fig. 1). Similar to the *Sun + Sky* and *Clear Bottom* chambers, we also used Propafilm® to cover up the top and bottom of the *Full Clear Chamber*. The internal parts of this chamber were covered with Teflon® tape to prevent water vapor absorption by the plexiglass, as recommended by LI-COR. This chamber required no adjustments (screws, seals, glue etc) because it perfectly set up onto the LI-6400 sensor head like the other one.

While in the 6400-02B leaf chamber leaf temperature (°C) was measured with a thermocouple, in the *Combination* and *Full Clear* chambers it was estimated by the energy balance method (Licor 2004). For the air temperature (°C) there were no differences in the manner of measurements, and it was measured by the internal sensor of the gas exchange system for the three chambers. The leaf vapor pressure deficit (kPa) was also measured for the three chambers in all days of measurements.

Measurements were performed at 9h, 12h, 14h and 16h, on cloudless clear days, during the spring (October) of 2012. The $CO_2$ assimilation ($A$; µmol m$^{-2}$ s$^{-1}$) and stomatal conductance (**$gs$**; mol m$^{-2}$ s$^{-1}$) were determined, using one leaf chamber per day of measurements, since it was not possible to use the three chambers on the same day. The transpiration rate ($E$; mmol m$^{-2}$ s$^{-1}$) and intercellular $CO_2$ concentration ($Ci$; µmol mol$^{-1}$) were also registered, but these parameters were not directly involved in the objectives of the study.

When using the LED *source*, the light source was set to provide 1600 µmol m$^{-2}$ s$^{-1}$ of photosynthetic photon flux density (PPFD), which is the PPFD that returns saturating values of $A$ for *S. camporum* leaves (Habermann *et al.* 2011). However, the natural PPFD

intercepted by leaves enclosed within the other leaf chambers was determined by an external quantum sensor (LI-190; LI-COR, USA), which was removed from the Irga's sensor head and placed close to the leaf surface, without disturbing its natural orientation during measurements.

**Leaf angle**

The petiole angle in relation to the horizon was measured according to Arena *et al.* (2008). A wire was positioned between the petiole and a ruler with a water level, which represented the horizon. The curvature radius formed by the wire copied the petiole angle, which was then measured with a goniometer. We separated vertical from horizontal leaves using the same parameters used by Habermann *et al.* (2011), and leaves showing angles between 0 and 20° were considered horizontal, whereas those exhibiting more than 60° were considered vertical leaves.

**Data analysis**

A one-way analysis of variance was performed between the three leaf chambers (*combination chamber*, *full clear chamber* and LED *source*), testing the leaf temperature and *A* values obtained at 9h, 12h, 14h and 16h. Therefore, for measurements obtained when light was intercepted by the adaxial, abaxial and both leaf surfaces in vertical and horizontal leaves, at each time of the day, mean results were compared by the Tukey's test ($\propto = 0.05$) to calculate the least significant difference between the three leaf chambers.

**Results**

The leaf temperature did not differ when measured or estimated with the different leaf chambers, regardless of the leaf type or time of the day (Table 1). The same was observed for the air temperature (Table S1 on supplementary data) and for the leaf vapor pressure deficit ($VPD_L$, Table S2).

Higher PPFD reached the adaxial side of horizontal leaves (Fig. 2B; ~1200 μmol $m^{-2}$ $s^{-1}$) in relation to when intercepted by the adaxial side of vertical leaves (Fig. 2A; ~700 μmol $m^{-2}$ $s^{-1}$), mainly at 9h and 12h. At 14h and 16h, sunlight was low and similar between leaf types (Fig. 2A, B). When sunlight was intercepted by the abaxial leaf side (~80 μmol $m^{-2}$ $s^{-1}$), the PPFD was considerably lower than when intercepted by the adaxial leaf side, regardless of the leaf chamber and leaf types (Fig. 2C, D).

When light was intercepted by both leaf surfaces of vertical leaves, *A* values measured with the *Full Clear Chamber* were the same as when measured with the *Combination Chamber* (Fig. 3A). However, for horizontal leaves, *A* values measured with the *Full Clear Chamber* were higher at 9h when compared to measurements obtained with the *Combination Chamber* (Fig. 3B).

As expected, the LED *source* returned the highest values of *A* when both the adaxial (Fig. 3C, D) and abaxial (Fig. 3E, F) leaf sides of vertical (Fig. 3C, E) and horizontal leaves (Fig. 3D, F) were exposed to the artificial light.

When light was intercepted by the adaxial side of vertical leaves, the *Full Clear Chamber* returned *A* values that were intermediate between those obtained with the LED *source* and *Combination Chamber*, although it showed lower values than LED *source* at the end of the day (Fig. 3C). For horizontal leaves, *A* values obtained with the three chambers were the same, except at 16h (Fig. 3D).

On the other hand, leaves assessed with the *Combination Chamber* returned *A* values that were lower than those measured with the LED *source* chamber, especially when light was intercepting the abaxial leaf sides of vertical and horizontal leaves (Fig. 3E, F). The *Full Clear Chamber* returned similar *A* values as those obtained with the LED *source* when light was intercepted by the abaxial side (Fig. 3E, F) of vertical and horizontal leaves, but especially at 9h and 12h.

With the exception of the *Full Clear Chamber*, $gs$ remained stable throughout the day decreasing with the natural decay of PPFD (data not shown). At 12h, *E* was high in both horizontal and vertical leaves when measured with the LED *source*. High *E* values were also found when measured with the *Full Clear Chamber* when light intercepted both sides of horizontal leaves, especially at 9h (data not shown). Intercellular $CO_2$ concentration was stable for both leaf types in the *Combination Chamber* ($250.4 \pm 20.8$ µmol mol$^{-1}$). The *Full Clear Chamber* showed high values at sunset when measured with light intercepting both leaf sides (data not shown).

The $CO_2$ assimilation was dependent on $gs$ regardless of the leaf chamber used to obtain data and of the leaf type and side (Fig. 4). However, when the abaxial side of both vertical and horizontal leaves were measured with the *Combination Chamber* we observed no correlation between $gs$ and *A* (Fig. 4B).

**Discussion**

According to our first hypothesis, we noted that different leaf chambers returned distinct values of $CO_2$ assimilation. This was especially confirmed when vertical leaves intercepted light by the adaxial leaf surface, evidencing that at 9h the LED *source* returned the highest *A* values while the *Combination Chamber* returned the lowest values and the *Full Clear Chamber* gave intermediate values (Fig. 3C). In fact, 9h-11h seems to be the

best time of the day to obtain the highest photosynthetic capacity, mainly in *S. camporum* (Feistler and Habermann 2012). For horizontal leaves, however, when intercepting light by the adaxial leaf surface *A* values were variable and similar between chambers. On the other hand, the role of the abaxial leaf side became paramount when analyzing the different capacity of chambers in returning *A* values. Although the LED *source* returned the highest *A* values in vertical leaves when intercepting light by the abaxial side, this chamber also demonstrated to be consistently superior (to the other chambers) from 9h to 16h, but mainly after noon (Fig. 3E). For horizontal leaves this response pattern (when measuring the abaxial side) could be also confirmed, although with a more inconsistent pattern of response (Fig. 3F).

These results highlight our second hipothesis that adaxial and abaxial sides show similar values of *A* when measured with the chamber possessing artificial light. In vertical leaves intercepting light by the adaxial side, the 8-4 µmol m$^{-2}$ s$^{-1}$ daily range (Fig. 3C) was similar to the 7-4 µmol m$^{-2}$ s$^{-1}$ range exhibited when light was intercepted by the abaxial side (Fig. 3E), and with some deviation in such ranges the same was true for horizontal leaves (Fig. 3D, F). Curves of *A*/PPFD measured on *S. camporum* using the same PPFD we used here demonstrated that for horizontal leaves the adaxial side returns higher *A* values when compared to the abaxial side, while for vertical leaves *A* values are similar between leaf sides (Habermann *et al.* 2011). Therefore, besides confirming our second hypothesis, which partly agrees with previous data (Habermann *et al.* 2011), the physiological role of the abaxial side to intercept light and result in $CO_2$ assimilation is reinforced.

The superiority of the LED *source* in returning the highest *A* values could be related with the stability of PPFD during the measurements, as well as with the spectrum (blue and red), which excite the chlorophylls of the photosystems with greater efficiency

(Zhu *et al.* 2010), in contrast to direct and/or indirect sunlight intercepting leaf sides when measured with the other two chambers. Even though the external PPFD was lower than 1600 μmol m$^{-2}$ s$^{-1}$ (set in the LED *source*) and unstable throughout the day (Fig. 2), when leaves were measured with the *Combination* or *Full Clear* chambers that allow sunlight interception by both leaf sides *A* values were comparable to those obtained with the LED source (Fig. 3A, B). Therefore, in contrast to our third premise, allowing illumination of both leaf sides while maintaining the leaf angle and conformation does not enhance the leaf capacity for carbon assimilation in this species. Leaves of *Olea europaea* when intercepting direct sunlight and reflected sunlight (by a mirror) on both leaf sides showed higher *A* values when compared to situations in which their leaves intercepted light on only one leaf side under the same PPFD (Proietti and Palliotti 1997). *Eucalyptus pauciflora* and *E. maculata* showed increased *A* values when equally illuminated on both leaf sides compared to when adaxial or abaxial illumination alone was applied (Evans *et al.* 1993). Although these authors corroborate the importance of both leaf sides on carbon assimilation, greater *A* is only achieved when both leaf surfaces are exposed to direct/reflected light or intentionally illuminated, as reported by these researchers. In the present study, we maintained the leaf angle and conformation, not intentionally exposing the leaf lamina to sunrays, and also considering scattered diffusive sunlight, usually intercepted by one of the leaf surfaces, which we believe to be a situation more close to the natural conditions. Therefore, using different leaf chambers, including clear chambers that allow light interception through every side for measuring *S. camporum* leaves returns *A* values that are not different from those obtained with the LED source.

We also show that *A* values are dependent on *gs* in *S. camporum* leaves, regardless of the leaf type (Fig. 4). This was already demonstrated by Habermann *et al.* (2011). These authors proposed that vertical leaves of this species could present increased light

use efficiency in terms of $CO_2$ consumption, as evidenced by augmented $A$ values obtained with a close gas exchange system (LI-6200, LI-COR). Our results show that despite the variable response pattern of $A$ throughout the day, vertical and horizontal leaves seem to have similar daily $A$ (Fig. 3A, B), and that totally clear chambers do not enhance $A$ in vertical leaves of this species. This also deny our fourth hypothesis that vertical and horizontal leaves could show distinct patterns of $A$ throughout the day. Although vertical leaves are strongly associated with photoprotection, especially in leguminous species (Caldas *et al.* 1997, Bielenberg *et al.* 2003, Pastenes *et al.* 2004b, Jiang *et al.* 2006, Arena *et al.* 2008), static vertical leaves of *S. camporum* could not be associated with photoprotection even under water deficit (Feistler and Habermann 2012). Therefore, it is difficult (or maybe impossible) to determine the ecological function of these static vertical leaves in this species.

**Acknowledgement**

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Zhu X.G., Long S.P., Ort D.R.: Improving photosynthetic efficiency for greater yield. – Annu. Rev. Plant Biol. **61**:235-261, 2010.

**Table 1.** Leaf temperature (ºC) measured (in LED *source*) and estimated (in *Combination Chamber* and *Full Clear Chamber*) on the adaxial surface of vertical and horizontal leaves of *Styrax camporum* using three different chambers of the gas exchange system.

| Leaf chambers | Vertical | | | |
|---|---|---|---|---|
| | 9h | 12h | 14h | 16h |
| *Combination Chamber* | 30.9 ± 0.76 | 32.7 ± 0.49 | 30.1 ± 0.31 | 29.4 ± 0.20 |
| *Full Clear Chamber* | 28.4 ± 0.61 | 29.6 ± 0.24 | 27.3 ± 0.03 | 27.4 ± 0.04 |
| *LED source* | 31.3 ± 1.03 | 34.2 ± 0.29 | 32.9 ± 0.66 | 31.4 ± 0.75 |
| | Horizontal | | | |
| | 9h | 12h | 14h | 16h |
| *Combination Chamber* | 31.3 ± 1.10 | 32.8 ± 0.47 | 30.2 ± 0.38 | 29.5 ± 0.32 |
| *Full Clear Chamber* | 28.2 ± 0.84 | 29.4 ± 1.02 | 27.4 ± 0.05 | 27.4 ± 0.04 |
| *LED source* | 31.0 ± 0.78 | 33.5 ± 0.79 | 32.8 ± 0.42 | 31.1 ± 0.47 |

Data shown as mean values (n = 5 plants) ± SD. For each leaf type (vertical and horizontal) at each time of the day, different letters represent significant differences between data obtained with the three leaf chambers by the Tukey test at 5% probability.
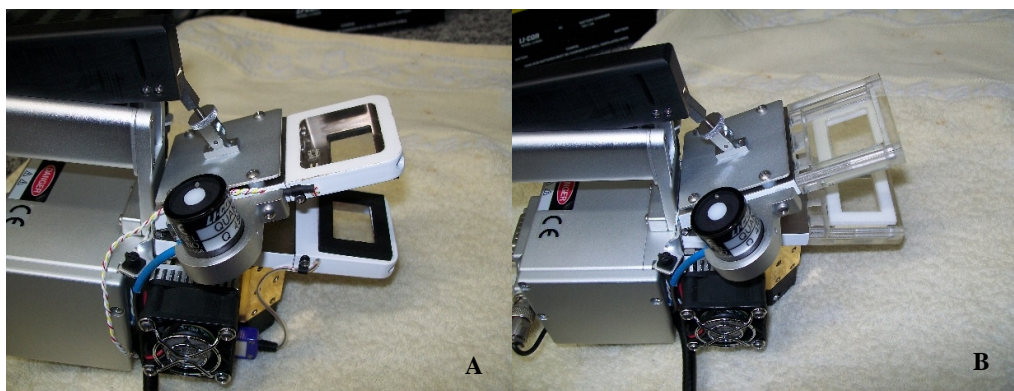
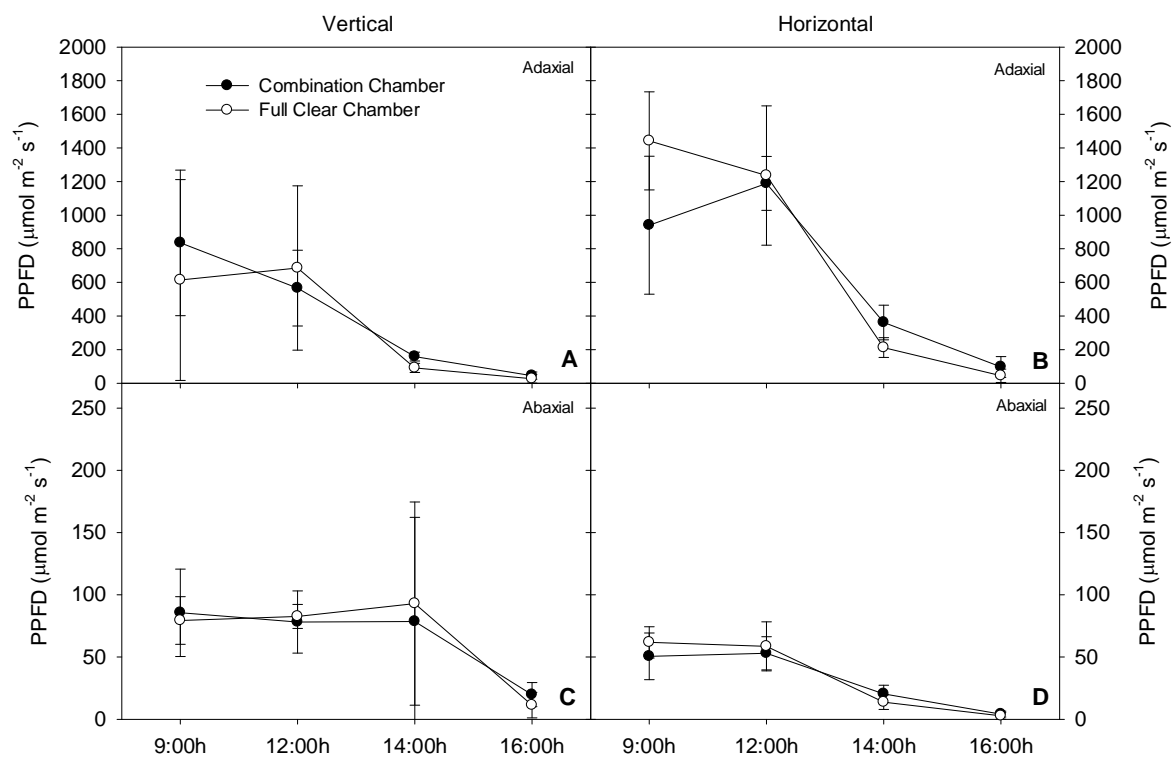**Fig. 1.** General views of the *Combination Chamber* (**A**) and *Full Clear Chamber* (**B**).

**Fig. 2.** Photosynthetic photon flux density (PPFD) measured at the leaf level on the adaxial (**A** and **B**) and abaxial (**C** and **D**) leaf surfaces of *S. camporum*
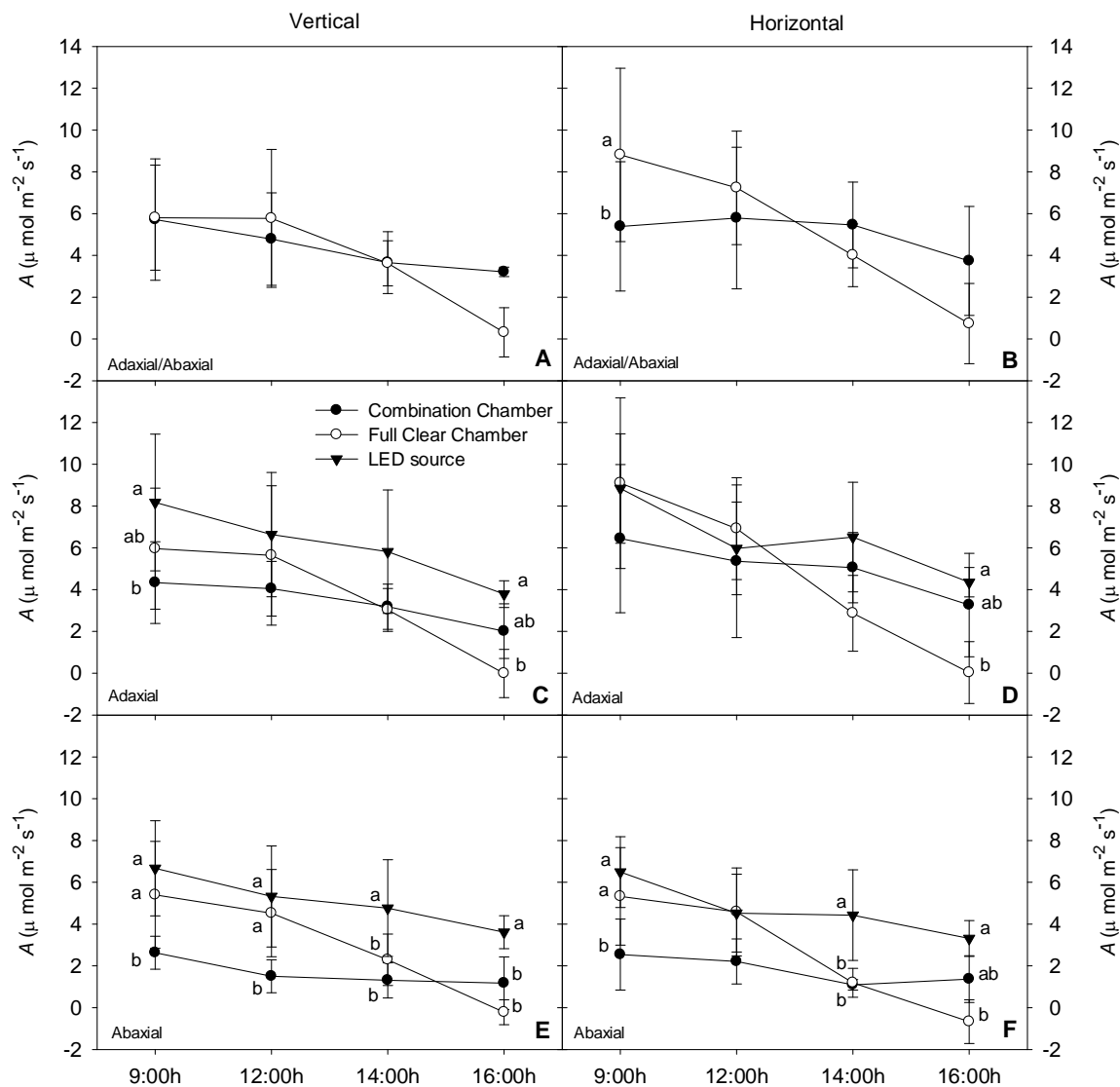
**Fig. 3.** Mean values (n = 5 plants) of $CO_2$ assimilation measured with light interception on both leaf sides (**A** and **B**), on the adaxial (**C**, **D**) and abaxial (**E, F**) sides of *S. camporum* leaves. For each leaf type, at each time of the day, absence of letters or same letters does not indicate differences between the leaf chambers by Tukey test at 5% probability. Bars = S.D.
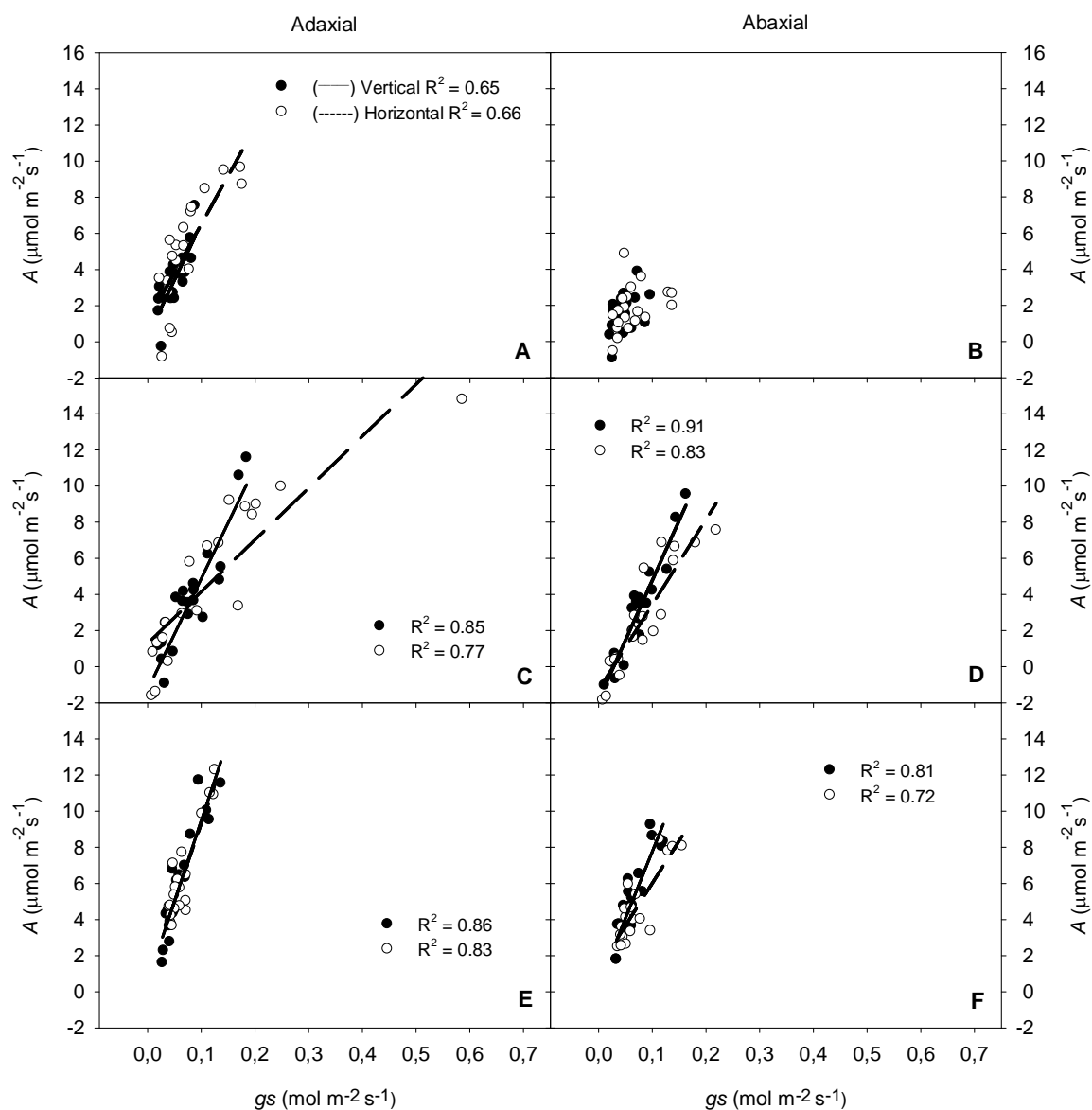
**Fig. 4.** Individual readings of $CO_2$ assimilation in response to the stomatal conductance for the adaxial (**A**, **C** and **E**) and abaxial (**B**, **D** and **F**) leaf surfaces measured with the *Combination Chamber* (**A**, **B**), *Full Clear Chamber* (**C**, **D**) and LED *source* (**E**, **F**). Only $R^2 > 0.5$ are shown.

**Supplementary data**

**Table S1.** Leaf vapor pressure deficit ($VPD_L$; kPa) measured on the adaxial surface of vertical and horizontal leaves of *Styrax camporum* using three different chambers of the gas exchange system.

| Leaf chambers | Vertical | | | |
|---|---|---|---|---|
| | 9h | 12h | 14h | 16h |
| *Combination Chamber* | 1.29 ± 0.07 | 2.07 ± 0.22 | 1.99 ± 0.10 | 2.30 ± 0.12 |
| *Full Clear Chamber* | 0.84 ± 0.14 | 1.14 ± 0.12 | 1.38 ± 0.22 | 2.09 ± 0.18 |
| *LED source* | 1.54 ± 0.23 | 2.47 ± 0.17 | 2.55 ± 0.26 | 2.55 ± 0.18 |
| | Horizontal | | | |
| | 9h | 12h | 14h | 16h |
| *Combination Chamber* | 1.30 ± 0.32 | 2.07 ± 0.15 | 1.96 ± 0.15 | 2.30 ± 0.17 |
| *Full Clear Chamber* | 0.77 ± 0.30 | 1.14 ± 0.16 | 1.47 ± 0.18 | 2.13 ± 0.20 |
| *LED source* | 1.45 ± 0.16 | 2.42 ± 0.16 | 2.49 ± 0.08 | 2.47 ± 0.13 |

Data shown as mean values (n = 5 plants) ± SD.

**Table S2.** Air temperature (ºC) during measurements in plants of *Styrax camporum* using three different chambers of the gas exchange system.

| Leaf chambers | Vertical | | | |
|---|---|---|---|---|
| | 9h | 12h | 14h | 16h |
| *Combination Chamber* | 29.3 ± 0.59 | 30.7 ± 0.30 | 28.7 ± 0.12 | 28.3 ± 0.14 |
| *Full Clear Chamber* | 29.6 ± 0.77 | 30.8 ± 0.73 | 28.6 ± 0.15 | 28.1 ± 0.20 |
| *LED source* | 28.5 ± 0.39 | 30.3 ± 0.23 | 29.1 ± 0.29 | 28.5 ± 0.12 |
| | Horizontal | | | |
| | 9h | 12h | 14h | 16h |
| *Combination Chamber* | 29.5 ± 0.92 | 30.5 ± 0.79 | 28.7 ± 0.17 | 28.3 ± 0.14 |
| *Full Clear Chamber* | 29.9 ± 0.95 | 30.7 ± 1.13 | 28.5 ± 0.13 | 28.0 ± 0.21 |
| *LED source* | 28.4 ± 0.30 | 30.1 ± 0.40 | 29.3 ± 0.22 | 28.5 ± 0.09 |

Data shown as mean values (n = 5 plants) ± SD.

# CHAPTER 2

# Vertical leaves promote high biomass accumulation in sugarcane: contribution of

# the abaxial leaf side

**Abstract**

The search for better crop production involves a range of different tools and techniques. Nevertheless the ideal production is still far from the theorized values. Here we analyzed the contribution of different leaf conformations in a sugarcane cultivar on $CO_2$ assimilation, chlorophyll fluorescence and biomass production. Cultivated in 50L-pots, five plants had their leaves artificially arranged in vertical position; five, in horizontal position, and five plants with unmanipulated leaves were used as control. Plants with vertical leaves showed more biomass accumulation, and increased $CO_2$ assimilation ($A$) was observed in these plants when the abaxial leaf side was illuminated either by natural or artificial light. Therefore, the vertical conformation seems to contribute to increased plant biomass due to an increased light use efficiency of the abaxial leaf side.

**Keywords:** $CO_2$ assimilation rate, leaf chambers, light use efficiency, *Saccharum* spp.

**Introduction**

Sunlight is the most important factor determining plant productivity (Lawlor 2001). Driving photosynthesis seems to be the main role of sunlight in plants, and the mechanisms involved in the rise of crop productivity have been recently studied (Long et al. 2006, Zhu et al. 2010), as this concern emerged from the necessity to provide food and energy in a world with growing human population (Evans 2013, Slattery and Ort 2015).

Plant responses to sunlight involve a pool of photochemical reactions and are influenced by a range of factors, such as water and $CO_2$ availability and leaf orientation. Several studies link sunlight interception by leaves, water deficit and $CO_2$ assimilation rates (*A*) (Pastenes et al. 2004a, Nakasuga 2013, Sales et al. 2015). Plants, in general, exhibit leaves with diferent inclination angles between the petiole and the horizontal plane, but most leaves can be considered horizontal – with less than 20° of inclination (Habermann et al. 2011). However, leaves of some species are oriented parallel to sunrays (paraheliotropism), intuitivelly avoiding sunlight, and this phenomenon is, therefore, commonly associated with a defense against photoinibition (Pastenes et al. 2004b, Jiang et al. 2006). This association between vertical leaf orientation and sunlight avoidance infer an almost unique understanding in the literature that such photoprotective and thermoprotective roles are able to diminish carbon losses and/or avoid stresses to the leaf (Caldas et al. 1997, Bielenberg et al. 2003, Pastenes et al. 2004b, Jiang et al. 2006, Arena et al. 2008).

However, it has been suggested that vertical leaf conformation is associated to an increased light use efficiency (Muraoka et al. 1998, Habermann et al. 2008, 2011), and a rise in carbon gain has been observed for vertical leaves (Falster and Westoby 2003). The ecological role of vertical leaves is hardly explored because photoprotective/thermoprotective hypotheses are rarely challenged, especially due to the

fact that same species do not present individuals with horizontal and individuals with vertical leaves, which could be eventually compared. In general, studies are performed in plants exhibiting paraheliotropism in leguminous species. However, there are plants with $C_3$ photosynthetic metabolism from the Brazilian savanna exhibiting static vertical and horizontal leaves, though on the same plant (Feistler and Habermann 2012). These authors demonstrated that vertical leaves of *S. camporum* are not associated to photoprotection, even under water deficit, and they raised the hypothesis that diferent leaf angles in this species could enhance the whole plant light interception efficiency.

In species showing both leaf types, it is not possible to assess the contribution of vertical leaves to the plant biomass. Monsi-Saeki theory and Boysen Jensen's work emphasize the importance of vertical leaf conformation for crop yields, although Boysen Jensen's argued that plant biomass discussion should shift to *A* (Hirose 2005). Accordingly, increased light availability at the bottom of a sugarcane canopy, as studied through different genotypes allowing distinct canopy sunlight interception, increases *A* at the bottom plant layer contributing to plant growth (Marchiori et al. 2010).

Less often attention is given to the photosynthetic contribution of leaf sides. Devices designed for gas exchange measurements are specific for or prescribed to measure the adaxial leaf surface and, usually, have a single light source to illuminate only one of the leaf sides.

Here we conducted a study with sugarcane in 50-L pots, and arranged their leaves into horizontal, vertical and also maintained a group of plants with unmanipulated leaves as control. We hypothesized that (1) vertical leaf orientation enhances biomass accumulation in sugarcane, and that (2) the abaxial leaf side play an important role on the carbon gain of plants with vertical leaves.

**Material and methods**

**Plant Material**

We used 15 plants of sugarcane (*Saccharum* spp.) cv. 'IACSP95-5000', which were obtained by planting individual nodes of mature cane stalks. The nodes were planted in 50-L pots (0.305 m in diameter and 0.585 m in height) containing a mix of oxisoil and a commercial organic substrate (Genebom®, Campinas, SP, Brazil) at 1:1 (v:v) proportion during the autumn, winter and spring (april-october) of 2015. Thirty days before planting, the soil inside pots was limed, according to the analysis of soil fertility. In addition, 60 days after emergence, a N-P-K (20:5:20; m:m:m) fertilization was performed, as recommended for this plant. Additionally, the plants received daily water supply.

**Study site and experimental description**

Pots were arranged in an open sky area (not inside a greenhouse), separated by 1.0 m from each other, with no trees around, and no control of irradiance, air temperature, wind or rain was used. Four wooden poles (3 m above-ground high) were fixed at the four edges where pots were placed, so that thick wires were fixed at the apex of each pole, forming a square. On this square, a net made of thin wires was constructed approximately 2.5 m above the pots.

After 30 days of planting, five plants were randomly selected to have their leaves arranged in a vertical position, five more plants had their leaves positioned horizontaly, and five plants with unmanipulated leaves were maintained as control. The study was conducted only with the main plant, suppressing the tillering by mechanical control. Vertical leaf position was arranged by attaching mini clothespin at the extreme end of leaves. The clothespins were tied with a nylon thread, which passed above the thin wire

net above the pots. At the end of nylon threads a slipknot hanged from the wire net were made and helped to maintain the leaf pulled and arranged in vertical position. As leaves grew, the nylon threads were shortened. The same procedure was taken to maintain leaves at the horizontal level, but instead of hanging the nylon thread these were tied to pieces of wood left on the floor, which prevented the leaf from becoming erect. The maintenance of this experimental design required daily adjustments and it was kept until 201 days after planting (DAP), when leaf area, stalk diameter and plant organ biomasses were evaluated. During the study, the number of leaves and plant height (from soil level up to the shoot apex) were registered at 39, 65, 103, 133 and 201 DAP.

To seek for the contribution of both adaxial and abaxial leaf sides to $CO_2$ assimilation rates, we used different leaf chambers when measuring gas exchange. One of the leaf chambers enabled artificial red (90%) and blue (10%) LED light interception by only one leaf side. This chamber also measured chlorophyll fluorescence. The other two chambers enabled sunlight interception on the adaxial and abaxial leaf sides. Therefore, we measured gas exchange in plants with vertical and horizontal leaves, as well as in control plants. Measurements were performed when only the adaxial, only the abaxial and when both leaf sides were illuminated. Gas exchange and chlorophyll fluorescence were measured at 60, 90, 120 and 180 DAP.

**Leaf angle measurement**

We assessed the inclination angle of leaf blades relative to horizon using a fine nylon thread attached to a transparent protractor. The protractor was held horizontally (0°), and the nylon thread indicating the leaf angle reproduced the inclination showed by the leaf.

**Leaf chambers**

We used a portable open-gas infrared analyzer, LI-6400xt (LI-COR, Lincoln, NE, USA). The leaf chamber enabling light interception by only one leaf side was the 6400-40 LCF (LI-COR, USA), which provided artificial red (90%) and blue (10%) LED light and also measured chlorophyll fluorescence. This light source was held on the opaque top of the 2 cm$^2$ leaf chamber. This leaf chamber will be named here 'LED *source*'.

We also used a combination of LI-COR chambers in which the *Sun + Sky Chamber* (LI-COR, USA) was set up on top of the 6 cm$^2$ leaf chamber and the *Clear Bottom Chamber* (LI-COR, USA) at the bottom of it, allowing natural irradiance through a propafilm® window (on a metal frame) to be intercepted by both leaf sides. This leaf chamber will be named here '*Combination Chamber*'. In this way, we also manipulated this *Combination Chamber*, so that when covering up (with a piece of cardboard) the top of this chamber natural light was intercepted by only the abaxial leaf side, and when covering up the bottom of this chamber, sunlight was intercepted by only the adaxial leaf side.

In addition, we made a third leaf chamber using plexiglass that will be named here '*Full Clear Chamber*'. This chamber was similar to the *Combination Chamber* (6 cm$^2$), but while metal is used for the *Combination Chamber* frame (2 x 3 cm), the *Full Clear Chamber* is entirely made of plexiglass, being totally tranparent. Similar to what was used in the *Sun + Sky* and *Clear Bottom* chambers, we also used Propafilm® to cover up the top and bottom of the *Full Clear Chamber*. The internal parts of this chamber were covered with Teflon® tape to prevent water vapor absorption by the plexiglass, as recommended by LI-COR. This chamber required no adjustments (screws, seals, glue, etc) because it perfectly set up onto the LI-6400xt sensor head.

**CO₂ assimilation and chlorophyll *a* fluorescence measurements**

Measurements were performed at consecutive dates between 9h and 11h on cloudless sunny days, using one mature fully expanded leave of each plant (vertical, horizontal and control) in its middle region. The $CO_2$ concentration, regardless of the leaf chamber used, was set to 390 µmol mol$^{-1}$. The photosynthetic photon flux density (PPFD) set in LED *source* was 1800 µmol photons m$^{-2}$ s$^{-1}$. The $CO_2$ assimilation rate (*A*) in response to PPFD (*A*/PPFD curves) was measured when the adaxial and abaxial leaf sides were illuminated by the LED light, and these curves are presented as suplementary results (Fig. S1).

Using the LED source (6400-40 LCF), the chlorophyll *a* fluorescence was measured in light-adapted and dark-adapted (with aluminum foils, for 30 min prior to measurements) leaves. The saturating light pulse was 7000 µmol m$^{-2}$ s$^{-1}$ during 0.7 s. We determined the maximal quantum yield of photosystem II (PSII), $F_v/F_m$ [$(F_m-F_o)/F_m$], where $F_v$, $F_o$ and $F_m$ are the variable, minimal and maximal fluorescence measured in dark-adapted leaves, respectively. We also measured the quantum yield of PSII, $\Phi_{PSII}$ [$(F_m'-F_s)/F_m'$] and electron transport rate, ETR [PPFD $A_{leaf}$ *fraction PSII* $\Phi_{PSII}$], where $F_m'$, $F_s$ are the maximal and steady-state fluorescence measured in light-adapted leaves; $A_{leaf}$ is the proportion of light absorptance by the leaf, and 0.84 was used here. The *fraction PSII* is the proportion of absorbed PPFD that reachs PSII, and 0.4 was used here (Baker 2008).

Additionally, we calculated ETR/$A_G$ ratio, where $A_G$ represents the gross photosynthetic rate defined as $A + R_d$, in which $R_d$ is the *A* value obtained in dark-adapted leaves (Wong et al. 2012). Photosythetic radiation use efficiency (PhRUE; *A*/PPFD) at saturating light was calculated but only for measurements performed with LED *source*.

**Biometric parameters**

Plant height was assessed with a measuring tape, the number of green leaves was counted, and the stalk diameter (cm) was assessed with calipers.

**Leaf area and partitioned dry biomass**

At the end of the study, the plants were cut just above the soil level, and leaf blade, stalk and sheath were separated. The leaf area, LA ($cm^2$) was measured with an area meter (LI-3100C, LI-COR, USA). For each plant, samples were oven-dried at 60ºC under forced air circulation, until constant dry mass (g).

**Data analysis**

A one-way analysis of variance was carried out between the three treatments (vertical, horizontal and control), testing LA, stalk diameter, number of leaves, plant heigth, leaf, sheath and stalk biomasses at 201 DAP. At 60, 90, 120 and 180 DAP *A* values, as well as photochemical parameters were tested between the three treatments when light was intercepted by the adaxial, abaxial or both leaf sides, using the three leaf chambers. Mean results accompanied by standard deviations were compared by Tukey test at 5% probability.

**Results**

Plants with vertical leaves showed leaf angles between 77 and 80º, while those from plants with horizontal leaves were between 7 and 12º. Control plants showed leaf angles with intermediate values between plants with vertical and horizontal leaves, but as these plants grew, their leaves became more erect increasing their leaf angles (Table 1).

Leaf area of plants with vertical leaves was 22% higher than that from plants with horizontal leaves, and was also larger than LA of control plants (Fig. 1A). On the other hand, stalk diameter was the same between the three treatments (Fig. 1B).

Plants with vertical leaves were taller than plants with horizontal leaves at 65, 103, 133 and 201 DAP, and control plants had intermediate height between plants with vertical and horizontal leaves (Fig. 2A). Plants with vertical leaves had more leaves than the other two treatments, but this was only observed at 201 DAP (Fig. 2B).

Leaf and sheath dry masses of plants with vertical leaves were higher than plants with horizontal leaves, and sheath dry mass was the same between plants with horizontal leaves and control plants (Fig. 3A, B). Leaf dry mass was the same between plants with vertical conformation and control plants (Fig. 3A). Although control plants exhibited intermediate stalk dry mass between plants with vertical and horizontal leaves, plants with vertical leaves showed 35% more stalk biomass in relation to plants with horizontal leaves (Fig. 3C).

The PPFD incoming to the adaxial leaf side oscillated between 1100 and 2000 $\mu$mol m$^{-2}$ s$^{-1}$ when measured with *Combination* and *Full Clear* chambers, regardless of the treatment that was evaluated (Fig. 4A,B). On the abaxial leaf side of plants from the three treatments PPFD varied between 50 and 150 $\mu$mol m$^{-2}$ s$^{-1}$ (Fig. 4C, D).

In all four evaluation dates, there was no difference in *A* values between the three treatments when the leaves were measured with light interception by both leaf sides, regardless of the leaf chamber used (Fig. 5A, B). The same response pattern was observed when the leaves were measured with light interception by the adaxial leaf side (Fig. 5C, D), which was also noted when the LED *source* exposed the adaxial surface to a constant red/blue irradiance (Fig. 6A). When the leaves were measured with light interception by only the abaxial leaf side using *Combination* and *Full Clear* chambers, plants with

vertical leaves showed higher $A$ in relation to plants with horizontal and to control plants (Fig. 5E, F); and when *Full Clear Chamber* was used, such difference was of at least 50% for every date (Fig. 5F). When using the LED *source* exposing artificial light to the abaxial leaf side it also returned higher $A$ values for plants with vertical leaves in relation to those with horizontal leaves, although control plants showed intermediate values (Fig. 6B). When $A/PPFD$ curves were performed with the LED *source* exposing light to the adaxial or to abaxial leaf sides, plants with vertical leaves also exhibited enhanced values (Fig. S1).

$F_v/F_m$ results were variable between evaluation dates when light was intercepted by the adaxial (Fig. 7A) and the abaxial (Fig. 7B) leaf sides. Plants with horizontal leaves showed the highest $\Phi_{PSII}$ (Fig. 7C) and ETR (Fig. 7E) when light intercepted the adaxial leaf side, while plants with vertical leaves showed a considerably higher abaxial $\Phi_{PSII}$ and ETR in relation to plants with horizontal leaves and to control plants (Fig. 7D, F).

When light was intercepted on the adaxial leaf side, plants with vertical leaves showed ETR/$A_G$ values lower than plants with horizontal leaves at 90, 120 and 180 DAP (Fig. 8A), and this parameter was not different between the three treatments when light was intercepted on the abaxial leaf side (Fig. 8B). PhRUE was the same among the three treatments when light intercepted on the adaxial leaf side (Fig. 8C), but it was higher for plants with vertical leaves when light intercepted on the abaxial leaf side (Fig. 8D).

**Discussion**

Our results show that plants with vertical leaves produce more biomass in relation to plants with horizontal leaves agreeing with our first hypothesis that vertical leaf orientation enhances biomass accumulation in sugarcane. When plants were harvested (201 DAP), plants with vertical leaves had a 22% larger total LA than plants with

horizontal leaves (Fig. 1A) probably because plants with horizontal leaves had 1-2 less leaves than plants with vertical leaves (Fig. 2B). This also reflected in leaf dry mass, which was 25% higher in plants with vertical leaves when compared to plants with horizontal leaves (Fig. 3A). Besides showing significantly higher leaf dry mass, plants with vertical leaves showed a 35% greater stalk dry mass in relation to plants with horizontal leaves (Fig. 3C), suggesting that vertical leaf orientation enhances stalk biomass.

Our data also point out why control plants showed, in general, intermediate results between plants with horizontal and vertical leaves. Control plants increased the leaf angle insertion along the experiment, exhibiting half of the leaf angle of plants with vertical leaves at 39 DAP but 85% of such leaf angle at 201 DAP (Table 1). This could be another indication that the leaf angle insertion may affect plant biomass and, consequently, plant yield. In fact, leaf angle insertion of sugarcane determines self-shading, which can reduce sunlight interception by leaves from the bottom layer of the plant canopy (Marchiori et al. 2010). In the present study, it would not be fair to discuss 'self-shading' because the pots (containing one plant each) were separated by 1.0 m from each other, a very contrasting condition to field studies (Bezuidenhout et al. 2003, Marchiori et al. 2010). Therefore, the present study explores a reason for higher biomass accumulation in plants with vertical leaves that does not emerge from (minor) 'self-shading' in these plants, but greater light use efficiency.

Among different plant species, sunlight interception in a plant's canopy can vary widely (Falster and Westoby 2003), including the distinct light incidence on leaf sides. Sunlight incidence between leaf sides of plants with vertical and horizontal leaves varied from sub-saturated to saturated values (Fig. 4A-D). Leaf angle insertions between plants with vertical and horizontal leaves and control plants (Table 1) represent the position of

leaf chambers at the moment of PPFD measurements. Although not statistically compared, greater leaf angles (plants with vertical leaves) promoted greater sunlight interception on the abaxial leaf side when measured with *Combination* and *Full Clear* chambers (Fig. 4C, D). Despite the fact that these assessments were made in the morning, the abaxial leaf sides of plants with vertical leaves were exposed to direct and/or diffuse sunlight, depending on the leaf's and plant's position, while the abaxial side of plants with horizontal leaves were exposed most of the time to diffuse light coming from the bottom, and this could explain the (low) PPFD incidence observed for the abaxial leaf sides (Fig. 4C, D) of plants with horizontal leaves. These observations are important when analyzing $CO_2$ assimilation rates, which depend directly on PPFD, especially in plants with $C_4$ photosynthetic metabolism (Sage and McKown 2006).

When both adaxial and abaxial leaf sides contributed to *A* values measured with *Combination* and *Full Clear* chambers (Fig. 5A, B), or even when using these chambers but allowing illumination on only the adaxial leaf side (Fig. 5C, D) the results were variable and not different between the three types of plants. However, the contribution of the abaxial leaf side to *A* arises as critical, mainly when measured with the *Full Clear* chamber (Fig. 5F). The importance of the abaxial leaf side to carbon assimilation can also be demonstrated when the abaxial leaf side of plants with vertical leaves was measured with *Combination* and *Full Clear* chambers but in a position that allowed sunrays to be intercepted *perpendicular* to the abaxial surface (in this case, the abaxial side was turned directly to the sun rays in the morning). This procedure returned *A* values that were the same from those obtained with the *adaxial* leaf side perpendicularly intercepting sunrays (data not shown). This observation is supported by the fact that, when measured with LED *source A* values between adaxial (Fig. 6A) and abaxial (Fig. 6B) sides of plants with vertical leaves were very similar. As one can observe, when measured with LED *source*

illuminating the abaxial side of plants with horizontal leaves and of control plants $A$ values were not as high as those obtained from plants with vertical leaves (Fig. 6B). In addition, although control plants increased their leaf angle insertion along the study, $A$ values obtained when illuminating their abaxial leaf side did not correspondingly increased. In fact, some innate responses of vertical leaves can be found in different species (Kitajima et al. 2005, Habbermann et al. 2011, Wong et al. 2012), indicating that a vertical position during the whole plant lifecycle is desirable. Therefore, despite vertical leaves might have somehow acclimated its photosynthetic responses, the importance of the abaxial leaf side in sugarcane seems to be paramount.

The adaxial leaf side of plants with horizontal and vertical leaves showed similar values of $F_v/F_m$ throughout the study (Fig. 7A). Although $\Phi_{PSII}$ and ETR measured on the adaxial leaf side were higher in plants with horizontal when compared to those with vertical leaves (Fig. 7C, E), it did not represent an increased photochemical performance in such a way to improve $CO_2$ assimilation, as $A$ was similar between the three types of plants when measured on the adaxial leaf side (Fig. 6A). Although returning lower $\Phi_{PSII}$ and ETR values when measured on the abaxial as compared to the adaxial leaf side, these variables were higher in plants with vertical when compared to those with horizontal leaves (Fig. 7D, F). However, the abaxial leaf side of plants with vertical leaves showed 25-50% higher $CO_2$ assimilation as compared to the abaxial leaf side of plants with horizontal leaves (Fig. 6B). This reinforces that the abaxial leaf side of plants with vertical leaves exhibited increased photochemical performance that was converted into carbon assimilation in the Calvin cycle. Therefore, besides accepting our second hypothesis that the abaxial leaf side plays a crucial role in plants with vertical leaves, these results strongly suggest that these plants increased their biomass production due to greater sunlight use efficiency. In fact, plants with vertical leaves showed PhRUE that was higher

than plants with horizontal leaves when measured on the abaxial leaf side, at 90, 120 and 180 DAP (Fig. 8D). Moreover, vertical leaf orientation also showed increased $A$ values in response to PPFD interception on either the adaxial (Fig. S1A) and abaxial (Fig. S1B) leaf side.

In the case of plants with horizontal leaves that showed elevated $\Phi_{PSII}$ and ETR in relation to plants with vertical leaves when these parameters were measured on the adaxial leaf side, it seems that such increased photochemical performance may have contributed to alternative electron pathways, as ETR/$A_G$ was higher in these plants (Fig. 8A). The raising of ETR/$A_G$ for many species under the same PPFD has been ascribed to an alternative electron flow (Wong et al. 2012). Although photorespiration is negligible in $C_4$ plants (Kakani et al. 2008), our results do not discard another electron sink as evidenced by the elevated ETR/$A_G$ in plants with horizontal leaves.

In the present study we did not observe any association between vertical leaf position and leaf heat avoidance, as the three plant types had similar leaf temperature when estimated on both leaf sides and with the three leaf chambers (Fig. S4, S5). Leaf heat avoidance is associated with vertical leaf position in leguminous species (Pastenes et al. 2004b, Arena et al. 2008) and other $C_3$ plants (Kalyar et al. 2003).

Although the leaf orientation is not the only factor to improve carbon gain in sugarcane, in the present study we show that vertical leaf orientation considerably contributes to the increase of plant biomass, including stalk biomass. The abaxial leaf side is of great importance in vertical leaves, as it enhances the carbon gain due to improved sunlight use efficiency.

**Acknowledgements**

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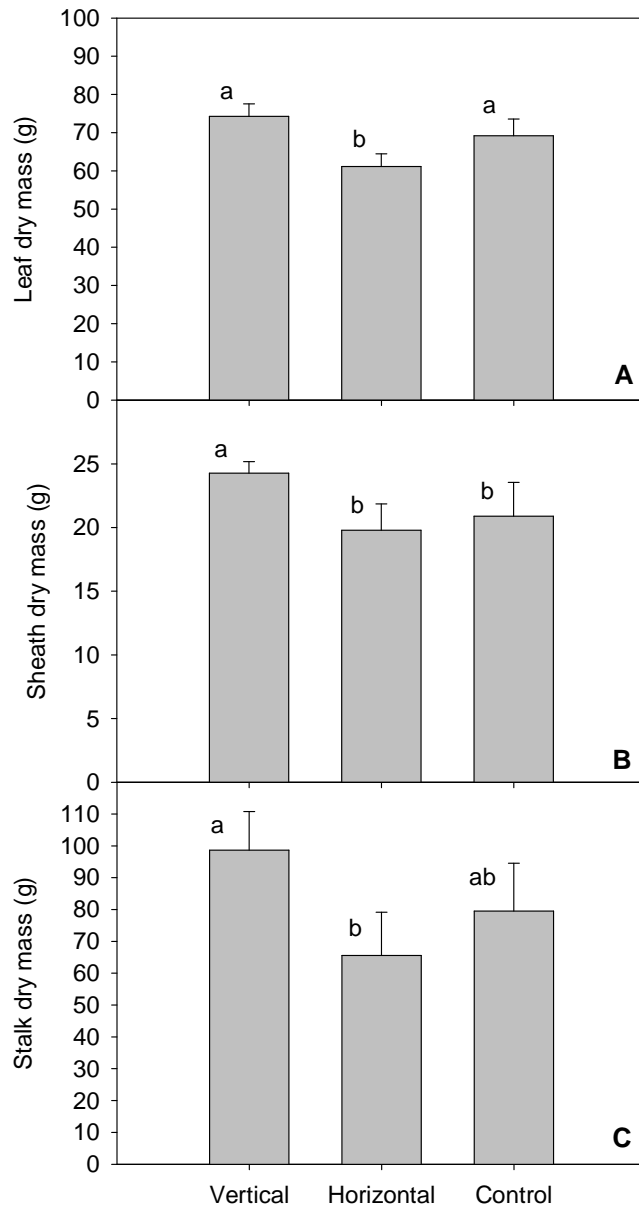
**Table 1.** Leaf angle insertion of sugarcane plants in which the leaves were arranged in vertical and horizontal positions, as well as unmanipulated leaves (control).

| Treatments | Days after planting | | | | |
|---|---|---|---|---|---|
| | 39 | 65 | 103 | 133 | 201 |
| Vertical | 79.0 ± 1.8 a | 79.8 ± 2.6 a | 81.3 ± 3.3 a | 80.0 ± 2.8 a | 77.2 ± 1.8 a |
| Horizontal | 12.5 ± 2.1 c | 8.0 ± 1.9 c | 7.1 ± 1.1 c | 7.6 ± 2.2 c | 8.1 ± 1.8 c |
| Control | 47.7 ± 0.9 b | 46.8 ± 4.7 b | 48.6 ± 4.2 b | 63.4 ± 1.9 b | 66.8 ± 3.8 b |

Data shown as mean of five replications (±SD). For each date, distinct letters indicate diferences between treatments by Tukey test at 5% probability
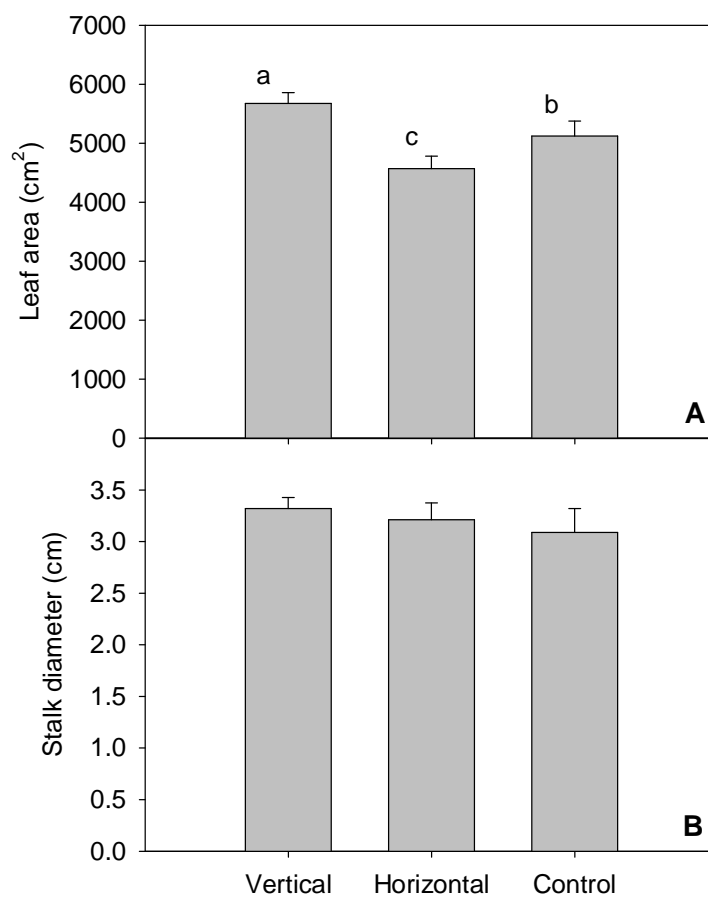
**Fig. 1.** Leaf area (**A**) and stalk diameter (**B**) of sugarcane plants in which the leaves were arranged in vertical and horizontal positions, as well as unmanipulated leaves (control). Columns represent mean values (n = 5 plants). Bars are SD. Absence of letters indicates no differences between treatments by Tukey test at 5% probability
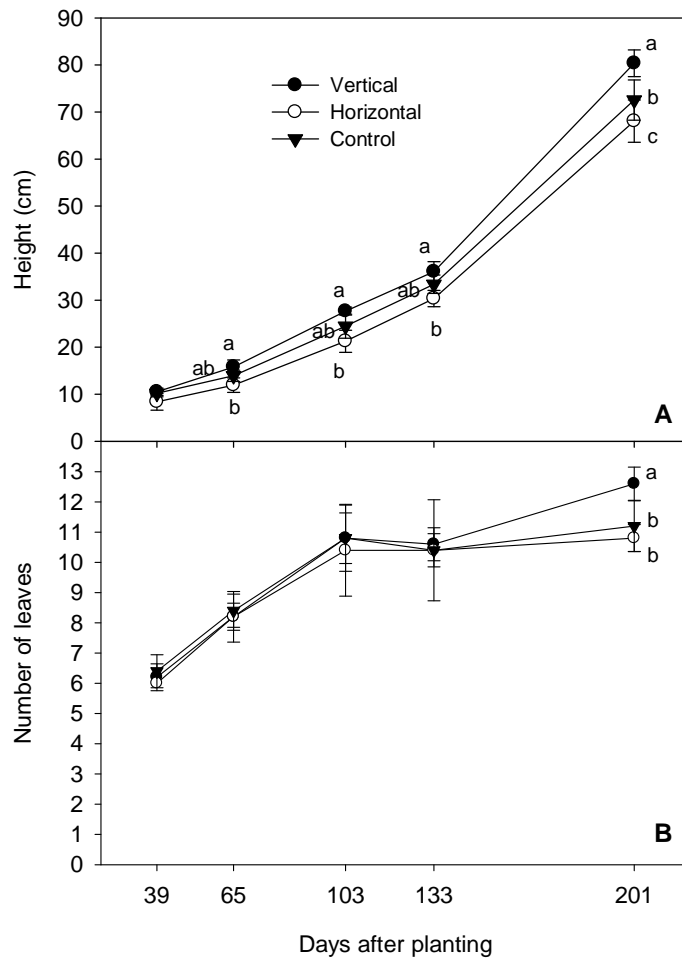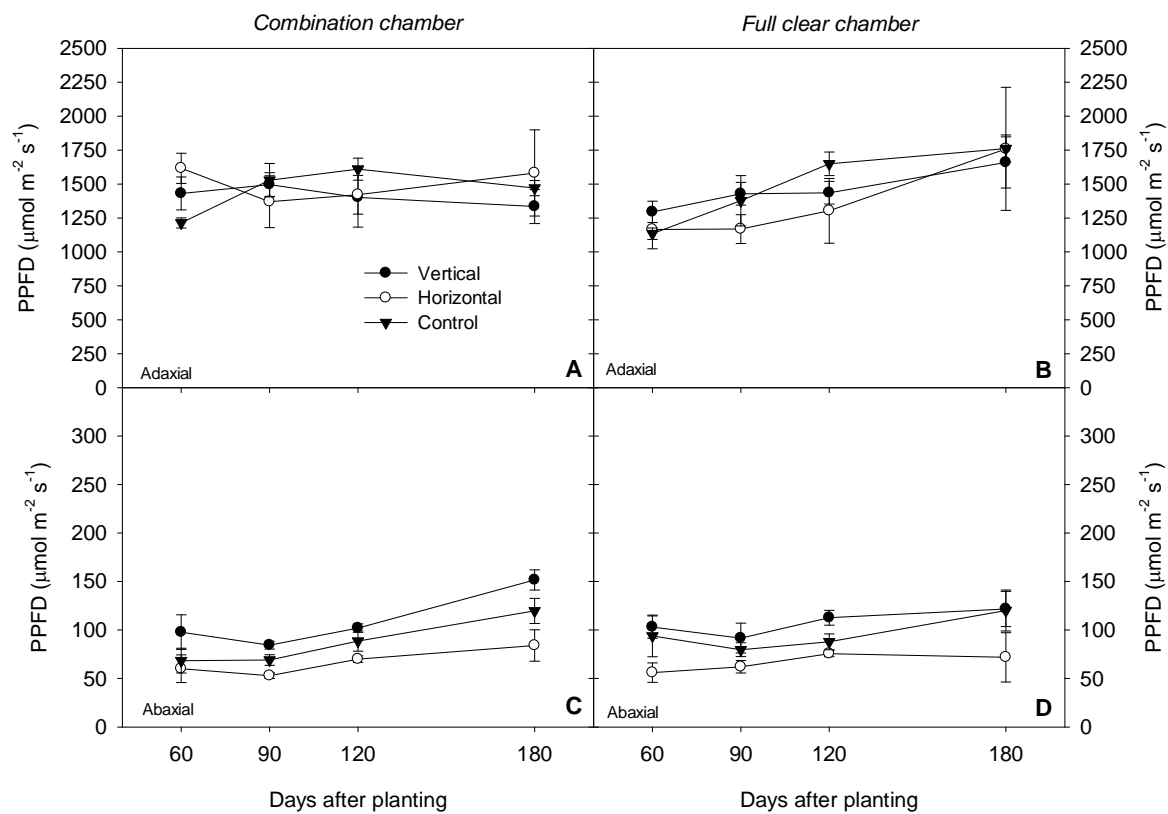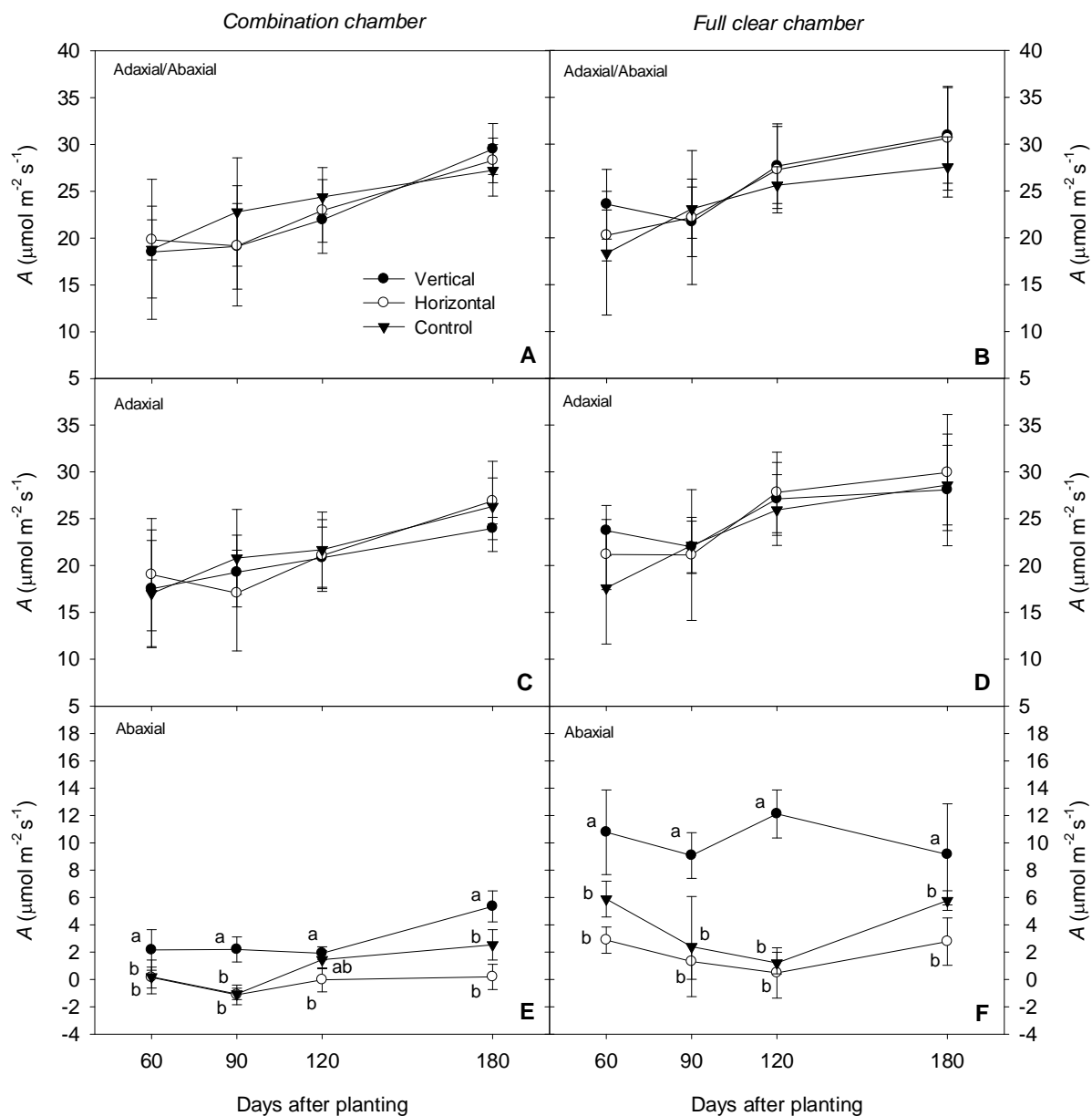
**Fig. 2.** Plant height (**A**) and number of leaves (**B**) of sugarcane plants in which the leaves were arranged in vertical and horizontal positions, as well as unmanipulated leaves (control). Each point represents mean values (n = 5 plants). Bars are ±SD. For each date, absence of letters indicates no differences between treatments by Tukey test at 5% probability

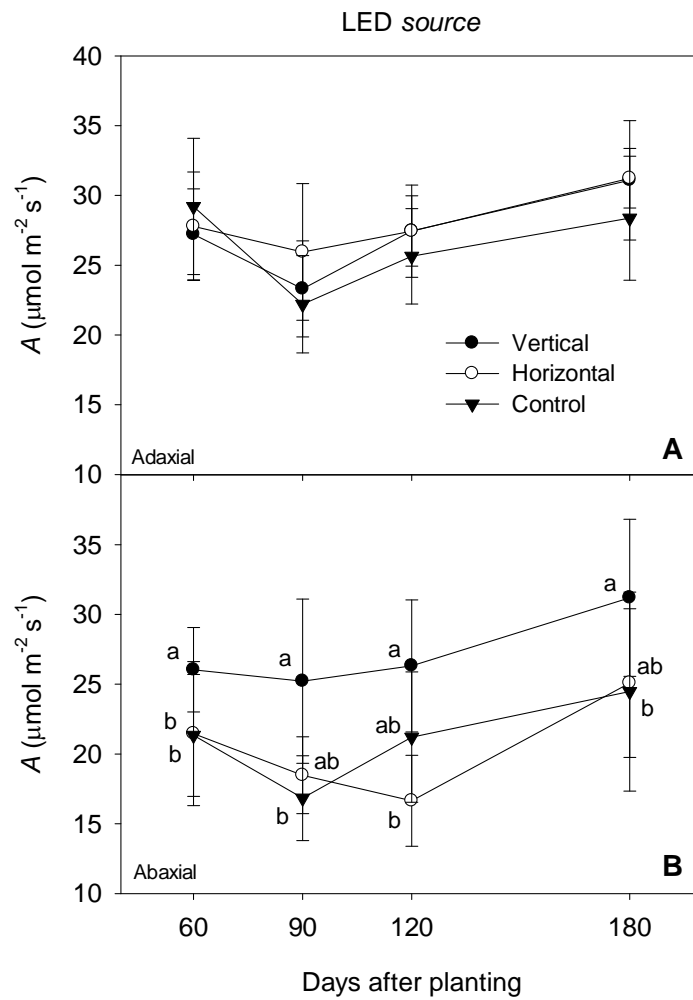**Fig. 3.** Biomass of organs of sugarcane plants in which the leaves were arranged in vertical and horizontal positions, as well as unmanipulated leaves (control). Columns represent mean values (n = 5 plants). Bars are SD. Different letters indicate differences between treatments by Tukey test at 5% probability

**Fig. 4.** Photosynthetic photon flux density (PPFD) measured on adaxial (**A, B**) and abaxial (**C, D**) leaf sides of sugarcane plants in which the leaves were arranged in vertical and horizontal positions, as well as unmanipulated leaves (control). Each point represents mean values (n = 5 plants). Bars are ±SD.

**Fig. 5.** $CO_2$ assimilation measured on both (**A**, **B**), adaxial (**C**, **D**) and abaxial (**E**, **F**) leaf sides of sugarcane plants in which the leaves were arranged in vertical and horizontal positions, as well as unmanipulated leaves (control). Each point represents mean values (n = 5 plants). Bars are ±SD. For each date, absence of letters or same letters indicates no differences between treatments by Tukey test at 5% probability
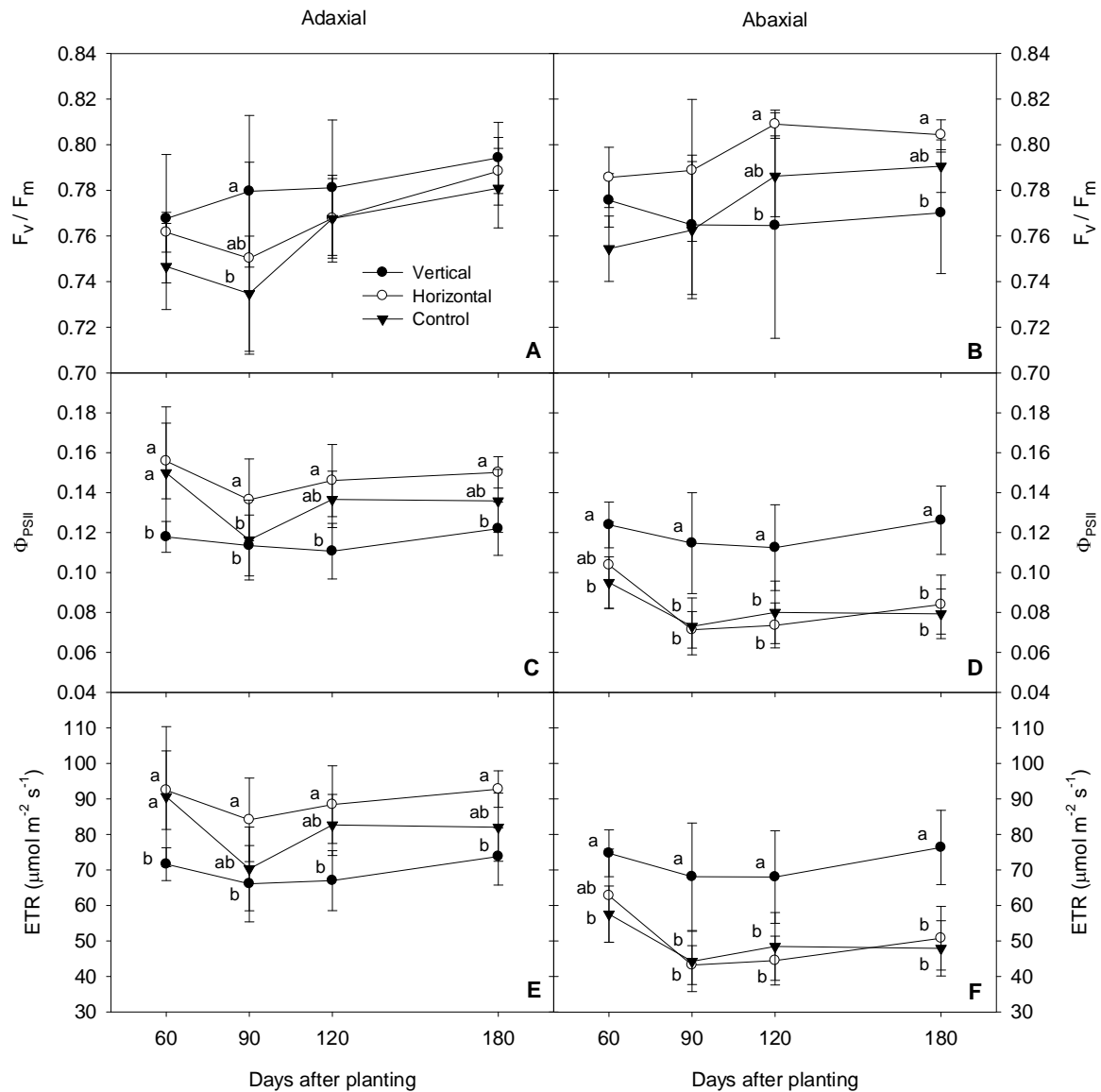
**Fig. 6.** $CO_2$ assimilation measured on adaxial (**A**) and abaxial (**B**) leaf sides of sugarcane

plants in which the leaves were arranged in vertical and horizontal positions, as well as

unmanipulated leaves (control). Each point represents mean values (n = 5 plants). Bars

are ±SD. For each date, absence of letters or same letters indicates no differences between treatments by
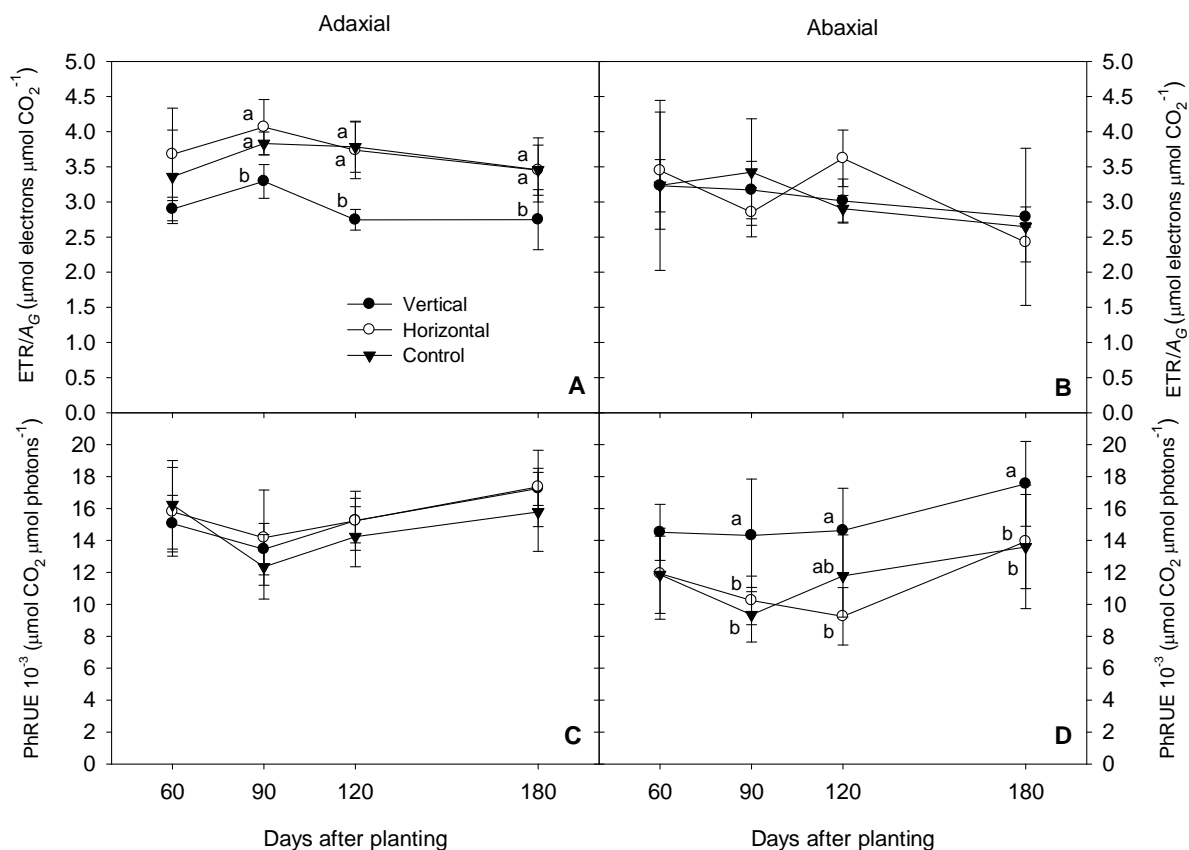
Tukey test at 5% probability

**Fig. 7.** Photochemical parameters measured on adaxial (**A, C, E**) and abaxial (**B, D, F**) leaf sides of sugarcane plants in which the leaves were arranged in vertical and horizontal positions, as well as unmanipulated leaves (control). Each point represents mean values (n = 5 plants). Bars are ±SD. For each date, absence of letters or same letters indicates no differences between treatments by Tukey test at 5% probability

**Fig. 8.** ETR/$A_G$ (**A**, **B**) and photosynthetic radiation use efficiency (PhRUE) (**C**, **D**) measured on adaxial (**A, C**) and abaxial (**B, D**) leaf sides of sugarcane plants in which the leaves were arranged in vertical and horizontal positions, as well as unmanipulated leaves (control). Eac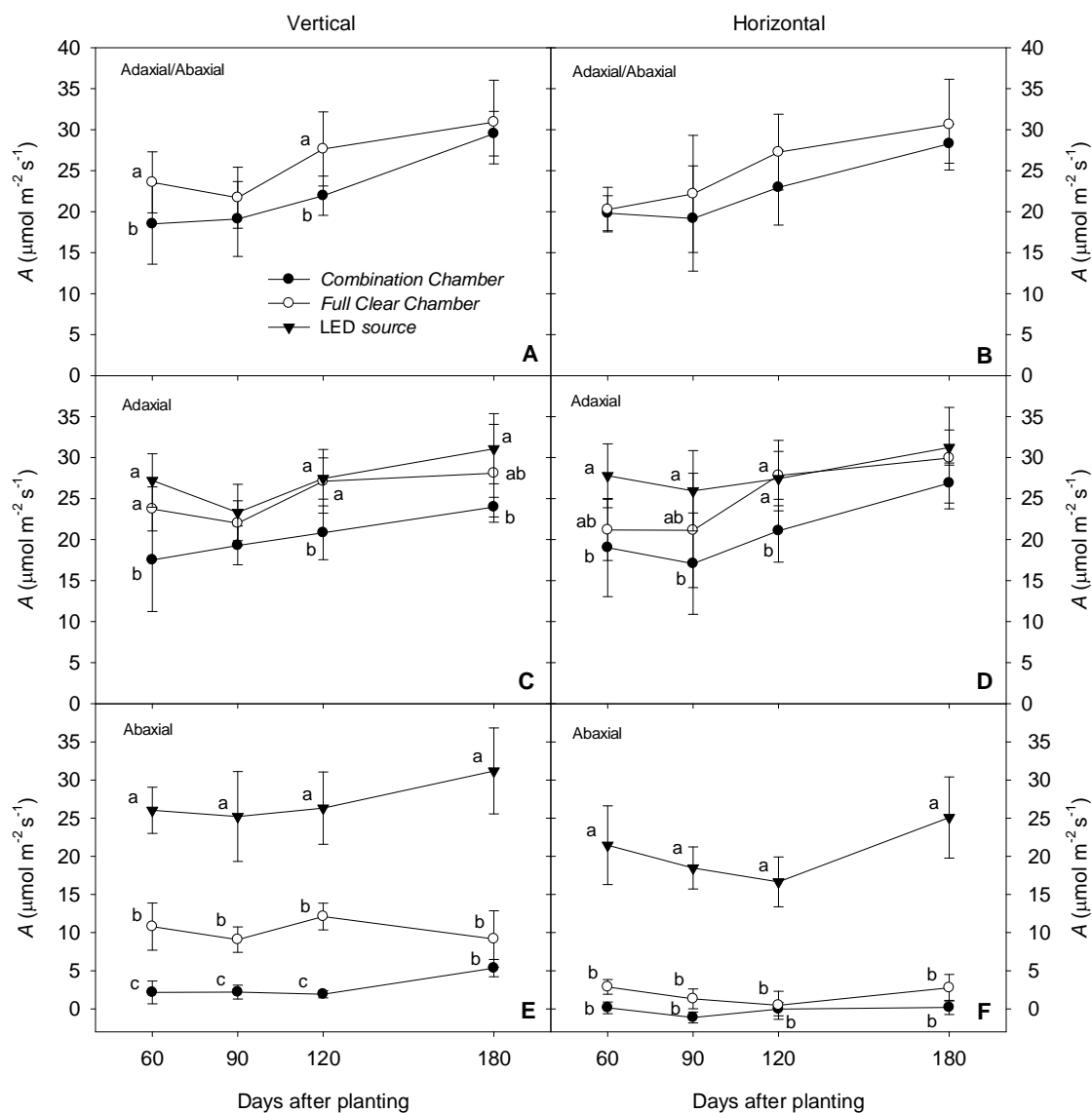h point represents mean values (n = 5 plants). Bars are ±SD. For each date, absence of letters or same letters indicate no differences between treatments by Tukey test at 5% probability

**Supplementary data**
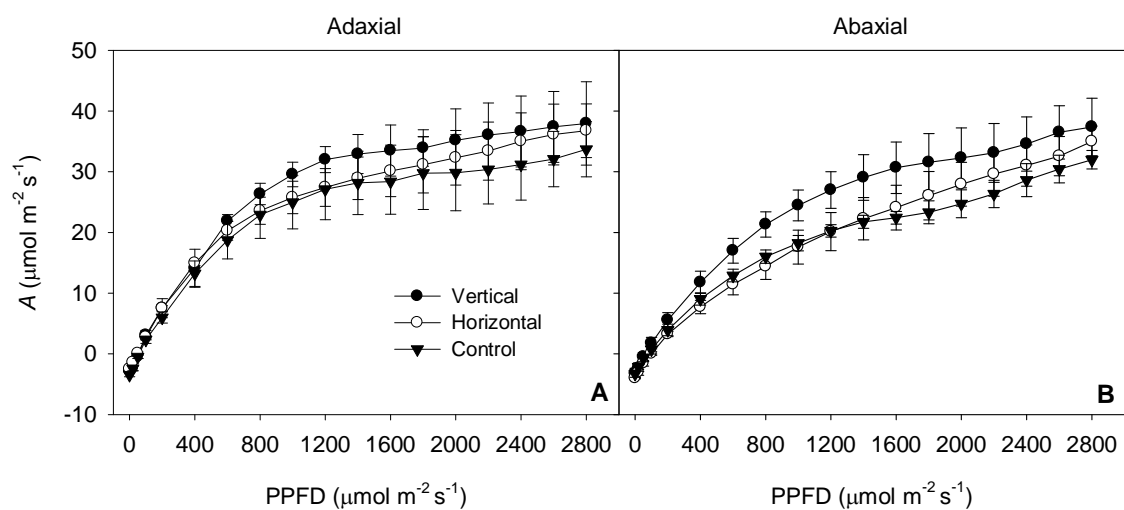


**Fig. S1.** $A$/PPFD curves measured at 190 days after planting on adaxial (**A**) and abaxial (**B**) leaf sides of sugarcane plants in which the leaves were arranged in vertical and horizontal positions, as well as unmanipulated leaves (control). Each point represent mean values (n = 3 plants). Bars are ±SD.

**Fig. S2.** $CO_2$ assimilation rates measured on both (**A, B**), adaxial (**C, D**) and abaxial (**E, F**) leaf sides of sugarcane plants in which the leaves were arranged in vertical (**A, C, E**) and horizontal (**B, D, F**) positions using leaf chambers that allow light interception on both leaf sides (*Combination* and *Full Clear* chambers) and on only one leaf side (LED *source*). Each point represents mean values (n = 5 plants). Bars are ±SD. For each date, absence of letters or same letters indicates no differences between treatments by Tukey test at 5% probability
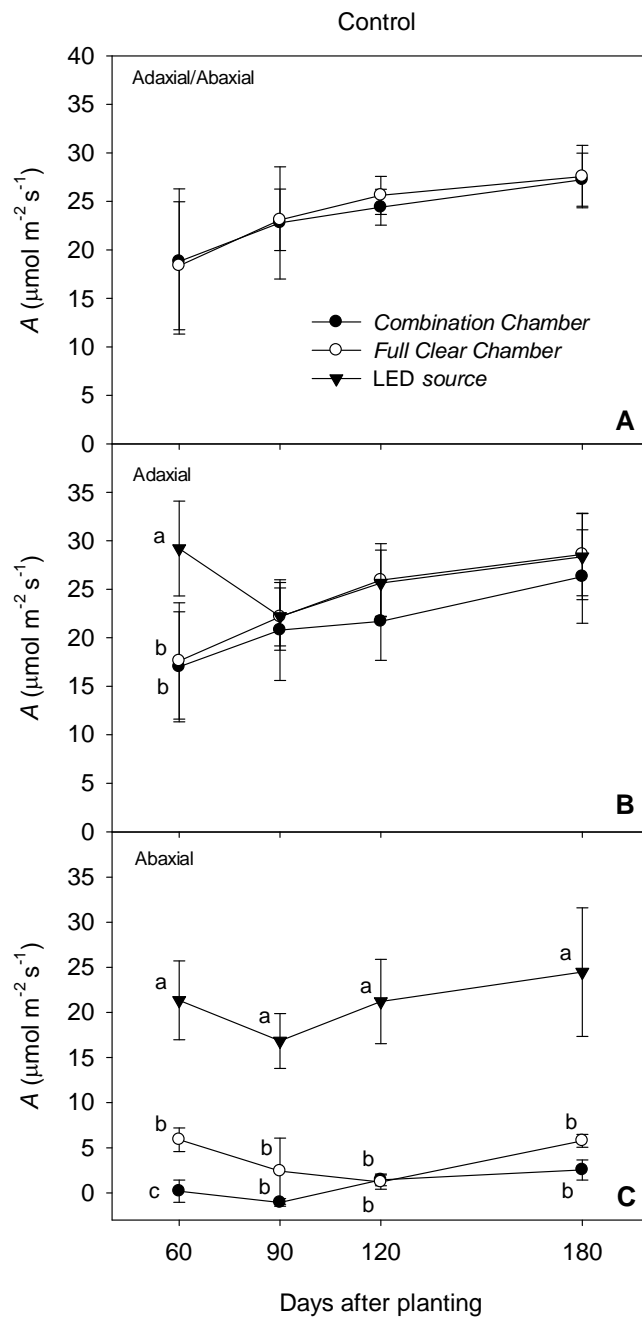
**Fig. S3.** $CO_2$ assimilation rates measured on both (**A**), adaxial (**B**) and abaxial (**C**) leaf sides of sugarcane plants using leaf chambers that allow light interception on both leaf sides (*Combination* and *Full Clear* chambers) and on only one leaf side (LED *source*). Each point represents mean values (n = 5 plants). Bars are ±SD. For each date, absence of letters or same letters indicates no differences between treatments by Tukey test at 5% probability
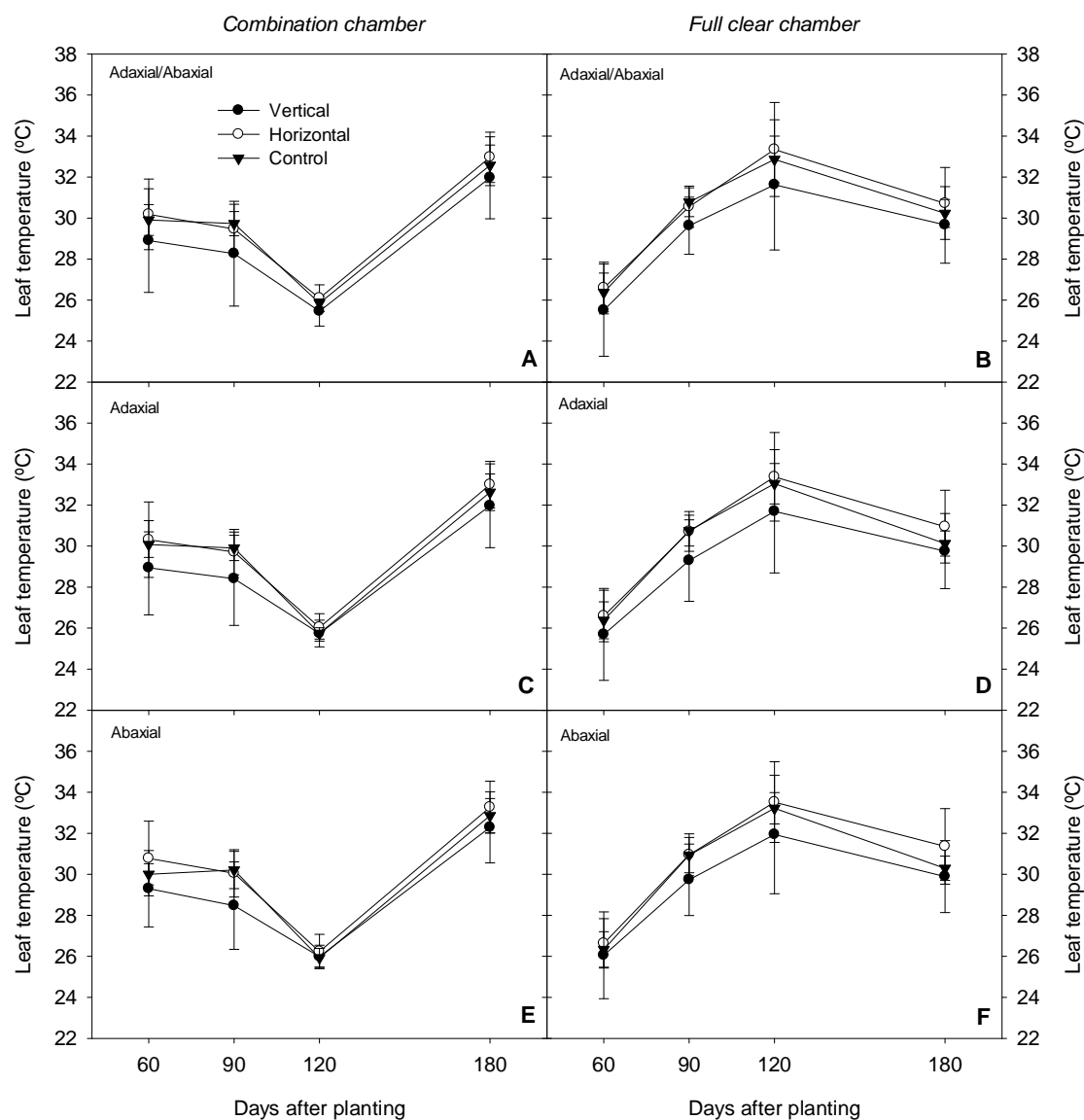
**Fig. S4.** Leaf temperature estimated on both (**A, B**), adaxial (**C, D**) and abaxial (**E, F**) leaf sides of sugarcane plants in which the leaves were arranged in vertical (**A, C, E**) and horizontal (**B, D, F**) positions using leaf chambers that allow light interception on both leaf sides. Each point represents mean values (n = 5 plants). Bars are ±SD. For each date, absence of letters or same letters indicates no differences between treatments by Tukey test at 5% probability
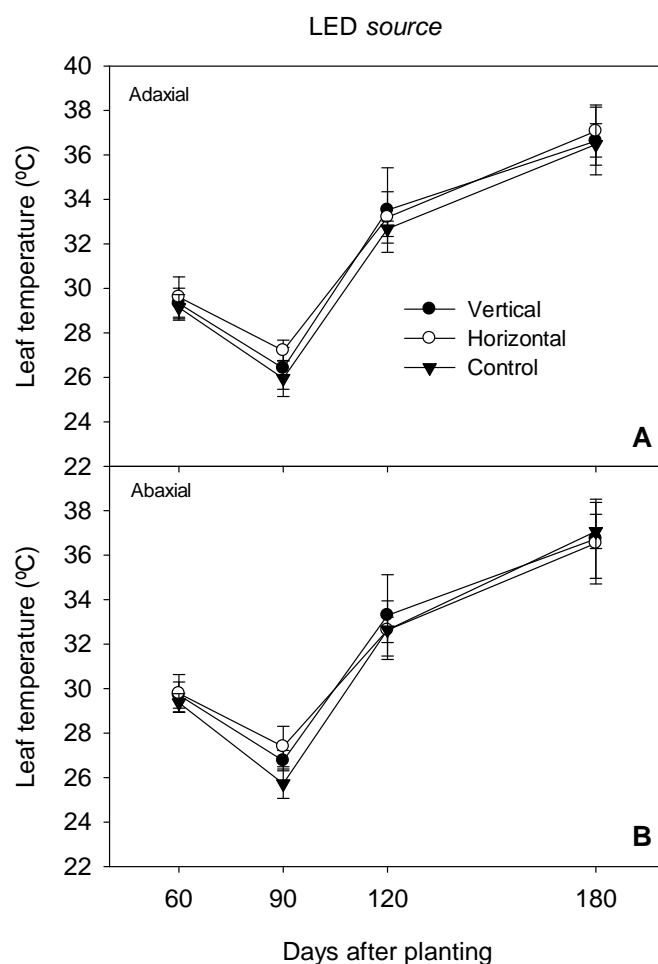
**Fig. S5**. Leaf temperature measured on adaxial (**A**) and abaxial (**B**) leaf sides of sugarcane plants in which the leaves were arranged in vertical and horizontal positions, as well as unmanipulated leaves (control) using a leaf chamber that allow light interception on only one leaf side. Each point represents mean values (n = 5 plants). Bars are ±SD. For each date, absence of letters or same letters indicates no differences between treatments by Tukey test at 5% probability