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<td>Author(s)</td>
<td>Ishida, Atsushi; Nakano, Takashi; Adachi, Minaco; Yoshimura, Kenichi; Osada, Noriyuki; Ladpala, Phanumard; Diloksumpun, Sapit; Puangchit, Ladawan; Yoshimura, Jin</td>
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Kyoto University
Effective use of high CO$_2$ efflux at the soil surface in a tropical understory plant

Atsushi Ishida$^1$, Takashi Nakano$^2$, Minaco Adachi$^3$, Kenichi Yoshimura$^4$, Noriyuki Osada$^5$, Phanumard Ladpala$^6$, Sapti Diloksumpun$^7$, Ladawan Puangchit$^7$ & Jin Yoshimura$^{8,9,10}$

Many terrestrial plants are C$_3$ plants that evolved in the Mesozoic Era when atmospheric CO$_2$ concentrations ([CO$_2$]) were high. Given current conditions, C$_3$ plants can no longer benefit from high ambient [CO$_2$]. Kaempferia marginata Carey is a unique understory ginger plant in the tropical dry forests of Thailand. The plant has two large flat leaves that spread on the soil surface. We found a large difference in [CO$_2$] between the partly closed space between the soil surface and the leaves (638 μmol mol$^{-1}$) and the atmosphere at 20 cm above ground level (412 μmol mol$^{-1}$). This finding indicates that the plants capture CO$_2$ efflux from the soil. Almost all of the stomata are located on the abaxial leaf surface. When ambient air [CO$_2$] was experimentally increased from 400 to 600 μmol mol$^{-1}$, net photosynthetic rates increased by 45 to 48% under near light-saturated conditions. No significant increase was observed under low light conditions. These data demonstrate that the unique leaf structure enhances carbon gain by trapping soil CO$_2$ efflux at stomatal sites under relatively high light conditions, suggesting that ambient air [CO$_2$] can serve as an important selective agent for terrestrial C$_3$ plants.

The geological record indicates that the C$_3$ land plants originated during the middle to late Ordovician period (450 to 440 million years ago) when atmospheric CO$_2$ concentrations ([CO$_2$]) were still very high (approximately 4% compared with 0.039% at present) and O$_2$ concentrations ([O$_2$]) in air were low (approximately 15% compared with 21% at present)$^{1,2}$. Although the down-regulation of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) under high [CO$_2$] is a well-known phenomenon$^3$, high [CO$_2$] and low [O$_2$] in the ancient air would have contributed to an increase in carbon assimilation rates ($A$) due to the kinetics of Rubisco. A meta-analysis of FACE (free-air CO$_2$ enrichment) experiments revealed that the average maximum carboxylation rates under doubled [CO$_2$] were −17% in C$_3$ crops and −4% in C$_3$ trees due to dawn-regulation. On average, the increase in light-saturated net photosynthesis under doubled [CO$_2$] was 13% in C$_3$ crops and 47% in C$_3$ trees$^4$. This finding may indicate that C$_3$ plants in the past exhibited increased carbon (C) gain and that more extensive C cycling occurred in forest ecosystems compared with the present era. During the Cenozoic era, atmospheric O$_2$ concentrations increased and atmospheric [CO$_2$] became largely depleted, with record minimum [CO$_2$] during the Oligocene/Miocene epoch (24 million years ago)$^5$. Since the advent of the Industrial Revolution, atmospheric [CO$_2$] has increased rather rapidly due to the modernization of human society and increasing reliance on coal and oil burning. In the photosynthetic CO$_2$-response curves of C$_3$ plants, the transition of the limitation from ribulose-1,5-bisphosphate (RuBP) carboxylation limitation to RuBP regeneration limitation is typically observed between ambient and doubled ambient [CO$_2$]$^6$. Thus, C$_3$ plants are constrained by the carboxylation limitation of RuBP in the present-day air [CO$_2$]. In contrast, photosynthesis in C$_4$ plants is not limited by low air [CO$_2$]$^7$ because these plants possess the appropriate enzyme (PEP carboxylase) and the specific anatomy in bundle sheath cells required to increase the CO$_2$ partial pressure around Rubisco sites$^8$. C$_3$ plants have evolved to improve plant carbon and water relations simultaneously during photosynthesis and to cope with declining atmospheric [CO$_2$] and increasing water demand$^{4,9,10}$. However, C$_3$ plants have not evolved carbon-concentrating mechanisms in their physiology and anatomy.
Even in present-day ecosystems, sites with high air [CO$_2$], such as forest floors$^{10}$ and volcanic vents$^{11}$ are observed. The high [CO$_2$] found on forest floors originates from the respiration of soil organisms and plant-root systems. Attention has been focused on the large contributions of sunflecks or sun patches to net C assimilation rates (A) in forest understory plants, indicating strong light limitation$^{12,13}$. However, the potential effects of rising [CO$_2$] on A in understory plants have rarely been evaluated. High [CO$_2$] should contribute to the survival of understory plants that experience reduced photosynthetic rates due to water stress$^{14}$. The stable carbon isotope ratios of understory plants indicate that these plants re-fix the efflux C in tropical$^{15}$ and cool-temperate forests$^{16}$.

High [CO$_2$] that originates from the soil surface dissipates rapidly due to diffusion and mass flow caused by wind. Although wind velocity is reduced near the understory, an extremely gentle breeze is sufficient to diffuse CO$_2$ from the soil surface$^{17}$. Therefore, for understory plants to effectively use this high soil-efflux [CO$_2$], they must trap CO$_2$ near the soil surface. In the present study, we report the discovery of an understory ginger plant, _Kaempferia marginata_ Carey (Zingiberaceae), which effectively traps soil-efflux CO$_2$ in the closed space between the soil surface and its leaves. This plant enhances photosynthesis by 45 to 48% under relatively high light conditions. It is a drought-deciduous, perennial herb found in tropical dry forests in Southeast Asia. Based on measurements of ambient air [CO$_2$], photosynthetic capacity, and the stable carbon isotope ratios in the lamina, we demonstrate that this ginger plant makes effective use of high [CO$_2$] on the forest floor.

**Results**

The ginger plant has a unique leaf structure; the individual plant has two flat leaves that spread on the soil surface, and the leaf edges are often curled downward to capture the air under its leaf blades (Fig. 1). The root system is small, indicating that this plant has a poor water uptake capacity. The uppermost height of a single leaf blade is only 24 mm above the ground surface on average and defines a relatively closed space between the leaf blade and the soil surface (Table S1). The stomatal densities were 1.6 mm$^{-2}$ and 20.9 mm$^{-2}$ on the adaxial and abaxial leaf surfaces, respectively, indicating that approximately all stomata face the soil surface. The distributions of leaf sizes and leaf morphologies indicate that as the leaf size increases with time, the leaf shape gradually becomes rounder (Fig. S1), contributing to an increase in the efficiency of trapping CO$_2$ efflux from the soil surface.

On a sunny day during the rainy season, the average daily [CO$_2$] was 412 μmol mol$^{-1}$ in the open air at 20 cm above the ground and 638 μmol mol$^{-1}$ in the space between the leaves and soil surface (Fig. 2). The maximum [CO$_2$] observed in the air space was greater than 1000 μmol mol$^{-1}$. Nevertheless, [CO$_2$] in the space largely fluctuated with temporal variations in wind velocity. The values (mean ± SD) of the stable carbon isotope ratios ($\delta^{13}$C) in the lamina were $-34.9 \pm 1.5\%$ in the ginger plants and $-29.1 \pm 1.5\%$ in the upper canopy leaves of woody plants in the dry evergreen forest (our unpublished data on woody plants). The low $\delta^{13}$C value in the ginger plants indicates high internal [CO$_2$] in the leaves during the day.

When the ambient-air [CO$_2$] was artificially increased from 400 to 600 μmol mol$^{-1}$, the A under near-light-saturated conditions (800 μmol m$^{-2}$ s$^{-1}$ PPF; photosynthetic photon flux) increased from 5.8 to 8.2 μmol m$^{-2}$ s$^{-1}$, a 45% increase (Fig. 3A). In contrast, under low light conditions (less than 70 μmol m$^{-2}$ s$^{-1}$ PPF), no significant increase was detected in A after elevating [CO$_2$] from 400 to 600 μmol mol$^{-1}$. We also measured ambient-air CO$_2$ response curves under 500 and 40 μmol m$^{-2}$ s$^{-1}$ PPFs. Both RuBP carboxylation and RuBP regeneration rates were reduced by the low PPF (Fig. 3B). When the ambient-air [CO$_2$] was increased from 400 to 600 μmol mol$^{-1}$, A increased by 48% under relatively strong sunlight (500 μmol m$^{-2}$ s$^{-1}$ PPF) and by 36% under reduced light (40 μmol m$^{-2}$ s$^{-1}$ PPF) conditions. The data indicate that a significant increase in A in response to elevated [CO$_2$] was more pronounced under unsilt conditions compared with shaded conditions. Sunflecks must thus cooperate with rising [CO$_2$] for enhancing of A$^{12,13}$.

**Discussion**

The data presented here indicate that the unique leaf structure of ginger plant enhances C fixation under high light conditions by effectively trapping high [CO$_2$] efflux in the relatively closed space between their leaves and the soil surfaces. In tropical forests, high termite activity at ground level prevents fallen leaves from covering the leaf surface of the ginger plants (Fig. 1A); the leaf litter layer typically remains fairly thin and does not persist for a long period of time. This may be a factor in explaining why the ginger plant has evolved to capture CO$_2$ efflux from soil respiration in tropical forests.

Another unique morphological characteristic of the ginger plant is the small root system (Fig. 1B). Large non-photosynthetic organs are found to have large respiration requirements$^{16,17}$. However, its small root system, the ginger plant has a very low CO$_2$ compensation point at the whole plant level, similar to leafy plants$^{14}$. Because of the small root system, the ginger plant can only grow during the favorable rainy season as an ephemeral plant. Another advantage is the high

**Figure 1** | An understory ginger plant, _Kaempferia marginata_ Carey, with a unique leaf structure in a tropical forest in Southeast Asia. (a) Field-grown plants, (b) A plant removed from the soil; two large leaves and a poor root system are evident.

**Figure 2** | Diurnal time variations in air CO$_2$ concentration at 20 cm above the ground (open circles) and in the air space between the leaf blade and soil surface (blue circles).
soil respiration during rainy seasons. In tropical dry forests in Thailand, where the ginger plant is a native species, the soil respiration rates become double during the rainy seasons. The mean soil respiration rate is approximately 7.67 μmol m⁻² s⁻¹ in the rainy season and approximately 3.63 μmol m⁻² s⁻¹ in the dry season.

A relatively high irradiance is required to effectively enhance A under elevated [CO₂] (Fig. 3B); light levels greater than approximately 6.4% of full sunlight appear to be required to maintain a population of the ginger plant (see Environmental description in Supplementary information). Under sunlit conditions, the risk of photoinhibition increases even in tropical climates, particularly in shaded plants at relatively high temperatures. However, in the ginger plant, xanthophyll-cycle dependent non-photochemical quenching (NPQ) appears to prevent chronic photoinhibition (Fig. S3). This unique adaptation to specific microhabitats is reflected by the plant distribution. In the tropical dry forests, the ginger plant is primarily located in the drought-deciduous forests with sparse tree cover and lightly shaded forest floors. In contrast, the ginger plant is exclusively located on the edges of dry evergreen forests with closed canopies.

The discovery of the morphological adaptation of the ginger plant is the first demonstration of the effective use of high CO₂ efflux from soil in understory C₃ plants. Their unique structure of this plant is characterized by large, flat leaves, thus earning the nickname “terrestrial water lily.” The shape delimits the space between the leaves and the ground surface (Fig. 1A). Plants with such an ideal leaf structure are rare even in the tropics. We suggest that the C₃ ginger plant evolved to cope with low atmospheric CO₂ by morphologically trapping high CO₂ efflux from the soil, whereas C₄ plants did so by physiologically concentrating CO₂ within the plant body. In adult trees of certain woody plants, the respiration rates per unit stem surface at breast height ranges from 1.2 μmol m⁻² s⁻¹ to 3.5 μmol m⁻² s⁻¹ considering the diameter of the CO₂ sensor probe was 18.5 mm, and the leaf diameter was greater than 100 mm. Because of without tube-absorbing and given a large leaf, CO₂ in the air space below the leaf could be directly measured (Fig. S4); it is possible that we did not completely avoid air leaks along the side of the prove, possibly resulting in an underestimation of CO₂.

While measuring diurnal leaf gas exchange, the diurnal variations in ambient air [CO₂] were simultaneously measured with thin-film capacitance CO₂ sensors (GM70, Vaisala, Helsinki, Finland) without tube-absorbing air. The CO₂ sensors were set at two heights: 1) 20 cm above the ground and 2) in the air space between the leaf blade and the ground surface in an individual plant with a relatively large leaf area. The diameter of the CO₂ sensor probe was 18.5 mm, and the leaf diameter was greater than 100 mm. Because of without tube-absorbing and given a large leaf, CO₂ in the air space below the leaf could be directly measured (Fig. S4); it is possible that we did not completely avoid air leaks along the side of the prove, possibly resulting in an underestimation of CO₂.

In the following days, to evaluate the interactive effects of light intensity and [CO₂] on A, we measured photosynthetic light responses (PPF-A curves) under different ambient air [CO₂] levels and photosynthetic ambient air CO₂ responses (Ca-A curves) under different light levels during the daylight hours (Ca refers to ambient air [CO₂]). To evaluate the average internal [CO₂] in leaves over a long time period, carbon isotope ratios in the eight laminae were examined with an isotope ratio mass spectrometer (DELTAV plus, Thermo Fisher Scientific Inc., Cambridge, UK). More detailed information is described in the supplementary information.


**Figure 3 | Photosynthetic responses under high CO₂ concentrations and high light conditions.** (a) Photosynthetic light response curves (PPF-A curves) at 600 μmol mol⁻¹ CO₂ (closed circles) and 400 μmol mol⁻¹ CO₂ (open circles), where PPF represents the photosynthetic photon flux at the leaf surface and A is net C assimilation rate. Bars indicate 1 SD. (b) Photosynthetic ambient-air CO₂ response curves (Ca-A curves) in a leaf blade at 500 μmol m⁻² s⁻¹ PPF (closed circles) and 40 μmol m⁻² s⁻¹ PPF (open circles), where Ca represents CO₂ concentration in outlet gas stream in the LI-6400, i.e., ambient air CO₂ concentration.


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Author contributions

A.I. and T.N. designed and carried out the major part of the field measurements. M.A, K.Y., et al. carried out the field works. A.I. and J.Y. wrote the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

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Supplementary Information

Title: Effective use of high CO₂ efflux at soil surface in a tropical understory plant

Authors: Atsushi Ishida, Takashi Nakano, Minaco Adachi, Kenichi Yoshimura, Nori yuki Osada, Phanumard Ladpala, Sapit Diloksumpun, Ladawan Puangchit, Jin Yoshimura

Environmental description and more detailed methods

Study site and plant materials
The study was located in the dry evergreen forest at the Sakaerat Environmental Research Station (14° 29’N, 101° 55’E, 563 m ASL), approximately 180 km northeast of Bangkok in Thailand. Mean annual temperature was 26.2°C, and mean annual rainfall was 1240 mm (Sakurai et al. 1998). There is a distinct dry season from November to March (Ishida et al. 2006). The soil is of sandstone origin and acidic (around 4.5 in pH), and has relatively poor nutrients with high porosity. Hopea ferrea Lanessan (Dipterocarpaceae) is the predominant tree with tall canopies (approximately 25-35 m high) in the evergreen forest. Details in landform and soil characteristics are shown in Pitman (1996) and Murata et al. (2009).

The examined ginger plant (Kaempferia marginata Carey, Zingiberaceae) is a drought-deciduous perennial herb. The plants are usually found near the roadside in the dry evergreen forests with dense canopies, and inside the drought deciduous forests with sparse canopies in the Sakaerat Environmental Research Station. Thus, the ginger plant seems to favor to a relatively light understory.

Measurements of leaf size distribution
We selected a population of the ginger plant found roadside in the dry evergreen forest. To examine the ontogenetic variations of leaf form, we measured the leaf area, leaf length and width, and the top heights of the leaf blades in 150 individual plants with contrasting plants size. The data in leaf shape and the top height are shown in Supplementary Fig. 1 and Supplementary Table 1, respectively.

Microclimate measurements
Photosynthetic photon flux (PPF) was measured with quantum sensors (LI-190SB, LI-COR, Lincoln, NE, USA) at an open place and two understory places around the center of a ginger plant population for three consecutive, relatively sunny days from July 15 to July 17 in 2008 in the middle of the wet season. PPF measurement at the open site was conducted on the top of a 45-m high scaffolding tower constructed near the study site, which exceeded the uppermost canopy in the forest. Ambient air temperature ($T_{air}$) and relative humidity (RH) in the understory were simultaneously measured with thermistor and thin-film capacitance sensors, respectively (Model 36355, Hioki-Denki, Nagano, Japan). These sensors were connected with small data loggers (Model 3631 or 3635, Hioki-Denki), and the data were minutely stored. These measurements in the understory were conducted at 20 cm above the ground near the center of the ginger population.
On a sunny day (July 15 in 2008), the diurnal variations in ambient air CO2 concentrations ([CO2]) were directly measured with thin-film capacitance CO2 sensors (GM70, Vaisala, Helsinki, Finland) without tube-absorbing air. The CO2 sensors were set at two heights: (1) 20 cm above the ground and (2) in the air space between the leaf blade and the ground surface in a lamina. To measure CO2 concentrations in the air space, we selected an individual plant with a relatively large leaf area to avoid air leak along the side of the censor probe (Supplementary Figure 4).

Environmental description
Supplementary Figure 2 shows the diurnal time variations in microclimate (PPF, air temperature, and relative humidity in air) in the examined population growing at a roadside in the dry evergreen forest and the diurnal variations in PPF at an open place. The daily total PPF at the study site relative to that of the open site was 6.4%. The understory light levels in tropical evergreen forest are approximately 1% of full sunlight or sometimes less than 1% (e.g., Ashton 1992). Because the ginger plants are not found in deeper-shaded sites in the evergreen forests, the light levels of approximately 6.4% relative to full sunlight appear to be required to maintain a population of the ginger plant.

More detailed methods in the photosynthetic capacity measurements
The leaf photosynthetic capacity in eight individuals was measured with an open, portable measurement system (LI-6400, LI-COR, Lincoln, NE). The measurement was conducted in six healthy, mature leaves. The leaf chamber with 6 cm² was used and the red-blue RED lamp unit was utilized as a light source. Photosynthetic light-response curves were measured on the same eight leaf blades under 600 and 400 µmol mol⁻¹ CO₂ in the inlet gas stream with LI-6400. The values of 400 and 600 µmol mol⁻¹ CO₂ were approximate and corresponded to daily mean air CO₂ concentrations at the 20 cm above and just below the leaf blades, respectively. PPFs were decreased stepwise from 800, 500, 200, 100, 70, 40, 30, 20, 10, 7, 3, to 0 µmol m⁻² s⁻¹. Light compensation points and apparent quantum use efficiencies were calculated from linear regressions under very low PPFs (from 0 to 10 µmol m⁻² s⁻¹). The mean leaf temperature during these measurements was 29.2°C which approximately corresponds to the daytime leaf temperature. Photosynthetic ambient air CO₂-response curves were measured on the same seven leaf blades under 500 and 40 µmol m⁻² s⁻¹ PPF with LI-6400. In the values that were exposed to sun-flecks and in those without sunflecks during the daytime, the values of 500 and 40 µmol m⁻² s⁻¹ PPF were used, respectively (see Supplementary Fig. 2). The [CO₂] in the inlet gas stream with LI-6400 increased stepwise from 0, 50, 100, 200, 300, 400, 500, 600, 700, 800, to 1000 µmol mol⁻¹.

More detailed methods in the diurnal variations of leaf gas exchange and chlorophyll fluorescence measurements
The diurnal time change in leaf gas exchange was measured with an open, portable measurement system (LI-6400, LI-COR, Lincoln, NE), from dawn to dusk on 15 July 2008. The measurement was conducted in eight healthy, mature leaves at approximately 30-minute intervals. The leaf chamber with 6 cm² was used and the top part of the chamber was sealed with a clear plastic plate to receive naturally incident PPF. The CO₂ concentration in the inlet gas stream within LI-6400 was adjusted at
600 μmol mol⁻¹, which approximately corresponded to the mean air [CO₂] just below the leaf blades (see Figure 2).

While measuring the leaf gas exchange, the diurnal time variations in chlorophyll a fluorescence were measured with a fluorescence meter (Mini-PAM, Walz, Effeltrich, Germany), according to Bilger et al. (1995). The fiber-optic cable was connected with the clear top-cover of the LI-6400 chamber, while the angle (60°) and the distance between the leaf surface and the fiber-optic cable were manually adjusted. Maximum fluorescence yield (Fₘ) and dark fluorescence yield (Fₒ) in photosystem II (PSII) were determined just before dawn. Just after the measurement of leaf gas exchange, we supplied a saturated-light pulse to the leaf surface. Maximum fluorescence (Fₘ') and steady-state fluorescence (F) in the light-adapted state of PSII were measured during the daytime. Chlorophyll fluorescence parameters were calculated, according to Genty et al. (1989). The potential maximum quantum yield of PSII (Fᵥ/Fₘ = (Fₘ-Fₒ)/Fₘ) was calculated from the dark-time measurements made before dawn. For each daytime measurement, the effective quantum yield of PSII (Φₚₛᵢᵡ = (Fₘ'-F)/Fₘ') was calculated. Assuming that photosystem I and II absorb equal amounts of light and the leaf absorbance of lamina is 0.84, the electron transport rate through PSII (ETR) was calculated as, ETR = 0.5 Φₚₛᵢᵡ 0.84 PPF (at the leaf surface). Non-photochemical quenching (NPQ = (Fₘ/Fₘ')-1) was also calculated. Data in chlorophyll fluorescence are showed in Supplementary Figure 3.

**Measurements of the nitrogen and stable carbon isotope ratio in lamina and the number and size of stomata**

After all measurements, we collected the leaves and then cut leaf discs with a borer. The leaf discs were oven dried (70°C, 72 hr) and weighed to determine leaf dry mass per unit leaf area (LMA). The total nitrogen (N) and carbon (C) contents within the leaf discs were measured with an N-C analyzer (Sumigraph NC-900, Sumitomo-Kagaku, Osaka).

To estimate the averaged internal CO₂ concentrations in leaves for a long time, the stable carbon isotope ratios (δ¹³C) in lamina were determined with an isotope ratio mass spectrometer (DELTA V Plus, Thermo Fisher Scientific Inc., Cambridge, UK). The δ¹³C values were expressed in delta notation relative to a PD Belemnite standard: δ¹³C = (Rₘ - Rₛₘₚₐₜₚₑ₅ₖ) 1000 (‰), where Rₘ is the ¹³C/¹²C ratios of the samples and Rₛₘₚₐ₅ₖ is the ¹³C/¹²C ratio of the standard.

The numbers and the pore length of stomata in the adaxial and abaxial leaf surfaces were determined by obtaining replicas of the surface of four healthy leaves with a celluloid plate (Universal Micro-printing, SUMP, Tokyo, Japan).
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Supplementary Figure 1: The ontogenetic variations in size and shape of leaf blades in 150 ginger individuals with different plant sizes. (a) The frequency of blade size of single leaves, and (b) the change of leaf shape (the ratio of length to width in each leaf blade) with leaf area.
Supplementary Figure 2: The diurnal time courses in microclimate at the understory during the successive three days from on 15 July to 17 July (the mid-rainy season) in 2008. Incident photosynthetic photon flux (PPF) at (a) an open place and (b) the understory (20 cm above the ground), and (c) air temperature ($T_{air}$) and relative humidity (RH) in air at an understory site.
Supplementary Figure 3: The relationships between photosynthetic capacity and photosynthetic phone flux (PPF) at the leaf surface. Data were obtained from the measurements of diurnal time courses in (a) net photosynthetic rates, (b) PSII quantum yield, (c) electron transport rates through PSII, and (d) Stern-Volmer non-photochemical quenching coefficient.
Supplementary Figure 4: The measurement of CO₂ concentrations in the air space between the leaf blade and the soil surface.

References