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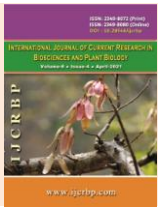

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The *in vitro* banana-leaf-fragment technique preserves the photosynthetic apparatus

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Abstract

In vitro studies of plant-pathogen interactions using leaf-fragments remains controversial compared to those studies under field conditions. The leaf-fragments technique, which predominantly uses benzimidazole in the culture medium to retard senescence, has been reported as reliable, fast, and inexpensive for analysis of aggressiveness and resistance in the *Mycosphaerella fijiensis-Musa* spp. interaction. However, no data have been published verifying whether *in vitro* banana leaf fragments maintain photosynthetic activity, which is a requirement for studying this interaction. In this study, maximum quantum efficiency of photosystem II, electron transport rate, transpiration, carbon dioxide exchange, light saturation point, and stomatal density were evaluated in *in vitro* leaf fragments of the Grand Nain banana genotype. Furthermore, the same parameters were also attained for leaves from plants in the field (during two seasons) and greenhouse conditions. The photosynthetic yield was constant during the experiment in leaf fragments with benzimidazole, and the photosynthetic rates on day 30 were similar throughout the whole experiment. This study supports that the banana-leaf-fragment technique as such protects the photosynthetic apparatus and then is suitable for studies on interactions such as that of *M. fijiensis-Musa acuminata*.

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Introduction

Bananas and plantains are important products worldwide, providing food and economic security in many countries of Latin America, the Caribbean, Africa, and Asia. Developed countries in Europe, North America, and other regions are the primary consumers, importing 93 % of the 20.2 million tonnes of banana, excluding plantain, exported globally in 2019 (FAO 2020). Because both banana and plantain crops are affected by fungal diseases, successful cultivar breeding depends on study of the plant-pathogen interactions. In this regard, the banana-leaf-fragment technique was developed to evaluate banana genotype resistance to the aggressiveness of *Mycosphaerella fijiensis* Morelet (El Hadrami, 2000), a fungal pathogen responsible for black Sigatoka disease. The development of this reliable, fast, cheap, and accurate method has been vital to studying this interaction. Many studies have used the detached leaf technique, since Yarwood described it in 1946, using exogenous agents to prevent tissue senescence (Osborne, 1967 cited by Mahapatra et al., 2015). Because leaf senescence is primarily the breakdown of chlorophyll, the colour change of the leaf indicates the physiological and biochemical changes associated with senescence (Woo et al., 2019). The main advantages of the detached leaf technique are to save space and host material, the low-cost of pathogen inoculum under study, the effortless and accuracy of the observations, and the possibility of maintaining the most prominent features and biochemical functions of plant leaves, including respiration, photosynthesis, and protein synthesis (Kamicoga, 2001).

The use of benzimidazole (*N, N'*-methylphenyl-*o*-phenylenediamine) has been one of the more successful treatments for tissue culture of wheat, rice, and millet (Person et al., 1957; Singh and Mishra, 1975; Mahapatra et al., 2015), as well as for banana leaf fragments maintained *in vitro* (El Hadrami, 2000). Addition of benzimidazole in the culture medium retards senescence of detached leaves and avoids respiratory rates increase. *In vitro* assays for resistance against *M. fijiensis* are effective using banana leaf fragments or detached leaves for rapid, massive screening of *Musa* spp. in breeding programs for black leaf streak resistance, before carrying out final confirmation trials under field conditions (Twizeyimana et al., 2007). Quantifying fungal damage by real-time polymerase chain reaction is important in plant resistance/susceptibility and pathogen aggressiveness studies. For this purpose, an efficient, artificial *M. fijiensis* inoculation protocol was developed using leaf fragments from mature banana plants grown under optimal physiological conditions (Abadie et al., 2008), but no physiological parameters have been measured in these fragments. Other authors are not convinced of the effectiveness of using leaf fragments in quantitative studies. For instance, when using banana leaf fragments to detect *M. fijiensis* and quantify its biomass, Arzanlou et al. (2007) observed significant data variation due to artificial inoculation and deemed the results as unreliable.

Environmental physiology in banana genotypes under field conditions for transpiration and CO_2 exchange evaluation has been thoroughly reviewed (Turner et al., 2007). However, no reports exist on photosynthetic parameters in banana leaf fragments under *in vitro* conditions. The primary question is if under these conditions the leaf fragments are photosynthetically active and thus a reliable model to study the *M. fijiensis*-*Musa* spp. interaction. The goal of this study was to document how an edible banana genotype, such as Grand Nain, behaves photosynthetically in *in vitro* (via leaf fragments) and to compare the same physiological parameters for plants in greenhouse and field conditions. Physiological measurements were maximum quantum efficiency of photosystem II (F_v/F_m), electron transport rate (ETR), transpiration, carbon dioxide uptake, light saturation point, and stomatal density.

Materials and methods

Plant material

The most important worldwide commercial Cavendish banana, the black Sigatoka-susceptible Grand Nain cultivar (*Musa acuminata* Colla, Cavendish subgroup, AAA), was selected to characterise the photosynthetic parameters in three conditions: *in vitro* leaf fragments, leaves of plants growing in the greenhouse, and leaves of plants growing in the field.

The plantlets obtained by organogenesis consisted of three phases: induction, multiplication, and rooting (Ma and Shii, 1972). These were removed from the flasks, washed with tap water to eliminate agar medium, their roots detached, transported to the greenhouse for transplanting on trays containing a mixture of soil and sunshine® (1:2, w/w), and covered with a dome of

translucent plastic during 7 days to allow 100 % of survival. Plantlets watered with Hoagland medium (Hoagland and Arnon, 1950) weekly, were transplanted to polybags with the same soil mixture as above after 60 days. The leaf fragments were cut from the plants with a height of 75 cm (approximately in 4 months) and the first two open leaves had a size of 30 cm long, and 16 cm width.

***In vitro* conditions:** Material for analysis consisted of leaf fragments (25 cm^2). Only the first and second healthy green leaves, previously disinfected with commercial bleach and rinsed three times with purified water, were used to obtain the fragments. They were placed in Petri dishes containing agar [0.5% (w/v) Merck, Damstadt, Germany] without and with 200 mg L^{-1} benzimidazole (Fluka, Steinheim, Germany), on the base of previously standardization, and incubated at 26-30 °C, with a 12 h photoperiod and 72 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) for one month.

Greenhouse conditions: Material for analysis consisted of the first green leaf from 4-mo-old plants growing in a greenhouse at the Yucatan Centre for Scientific Research (Centro de Investigación Científica de Yucatán; CICY) in Merida, Yucatan, Mexico. The greenhouse had an average temperature of 28 °C and a maximum photosynthetic photon flux density (PPFD) of 229 µmol m^{-2} s^{-1}. Physiological measurements were performed here in March and September.

Field conditions: Material for analysis consisted of the third-youngest leaf from one-year-old plants grown at the Uxmal Experimental Field Station of INIFAP in Yucatan, Mexico. Average annual rainfall at this location is 1072 mm, with a rainy season from June to November and an annual mean temperature of 25 °C.

Stomatal density

Stomata were counted in abaxial and adaxial leaf sections (0.3 mm^2) from plants grown under field and greenhouse conditions. Five counts of each leaf section, from the middle portion of the lamina near the leaf blade, were made using an Axioplan light microscope at 200x (Carl Zeiss, Gottingen, Germany).

Chlorophyll fluorescence measurements

Photosynthetic parameters were measured in the greenhouse and in the field in the dry and rainy seasons (n=5). Under *in vitro* conditions, parameters were measured at 1, 5, 15, and 30 days after they were placed on culture medium (n=10).

Maximum quantum efficiency of photosystem II (F_v/F_m) and electron transport rate (ETR) were obtained using a portable pulse-amplitude-modulated photosynthesis yield analyser (MiniPAM, Walz, Germany). The fibre-optic was kept at a constant distance of about 20 mm and an angle of approximately 60° to the upper surface of the leaf or leaf fragment. Measurements in the field and greenhouse were taken every 3 h during the day (from 06:00 to 18:00 h), and those in *in vitro* conditions were taken twice, in the dark (06:00 h) and light (10:00 h). The F_v/F_m was obtained as $(F_m - F_o)/F_m$ and PSII efficiency (Yield) as $(F_m' - F_t)/F_m'$, where F_m is maximum fluorescence at dawn, F_m' is maximum fluorescence in the day of light-adapted leaves, F_o is the yield of fluorescence of dark-adapted leaves, and F_t is the value of steady-state yield of fluorescence in the light (Maxwell and Johnson 2000). The ETR was obtained as PSII × PPFD × 0.5 × 0.84, where 0.5 assumes that both photosystems II and I are equally excited by the irradiance, and the factor 0.84 is the absorbance of the incident irradiance by the photosystems (Genty et al., 1989; Ritchie and Bunthawin, 2010). Under field conditions, light curves were performed on leaves to determine their light saturation points. Leaves were exposed to nine increasing PPFD ranks from 0–900 µmol $m^{-2}s^{-1}$.

Transpiration and CO_2 exchange

Gas exchange was measured in leaves of the same plants (both greenhouse and field) and in leaf fragments immediately after chlorophyll fluorescence measurements. All measurements were made using an infrared gas analyser (Portable Photosynthesis System LI-6400, Li-COR, Inc., Lincoln, USA) with air humidity and CO_2 concentrations similar to ambient levels. Water use efficiency (WUE) was calculated as CO_2 uptake/ transpiration rate (mol CO_2 m^{-2} per mol H_2O m^{-2}). Both chlorophyll fluorescence and gas exchange measurements were made by removing the leaf fragments from the medium (with or without benzimidazole) but returning them after the measurements.

Statistical analysis

Multiple-sample comparisons using Kruskal-Wallis tests and box and whisker plots were performed to

evaluate differences in stomatal density with the STATGRAPHICS Plus version 4.1 software package (Manugistic, Rockville, USA). To evaluate differences in photosynthesis and transpiration rates between the control and benzimidazole treatments for *in vitro* tissues, a repeated measurement analysis of variance (ANOVA) (P = 0.05) was done, followed by Fisher post-hoc tests. The factors considered were time of photoperiod (dark or light), days in *in vitro* conditions (1, 5, 15, and 30 days), and treatment (with or without benzimidazole), and the variables were photosynthesis, transpiration rate, yield, and ETR. Data were tested for normality and homoscedasticity. Transpiration rate data were normalised by 1/x. For F_v/F_m, a Mann-Whitney U test was used. Statistical analysis of the control fragments was made from day 1–15 for photosynthesis and transpiration because senescence occurred after this time.

Differences in ETR and Yield in the greenhouse and field were evaluated with Two-way ANOVA. It was considered time of day (6, 9, 12, 15, and 18 h), month (March and September), and season (dry and rainy) as factors, and when the variables were not normally distributed, a Kruskal-Wallis test was applied. One-way ANOVA was used to evaluate the differences in F_v/F_m, considering the month or season as factors in the greenhouse or field, respectively. Statistica 8 software (Statsoft, Inc. Oklahoma, USA) was used for all statistical analysis.

Results and discussion

Stomatal density

The Grand Nain genotype exhibited greater stomatal density on the abaxial epidermis than on the adaxial epidermis, under field and under greenhouse conditions. However, the greenhouse-cultivated plants exhibited higher stomatal density on the abaxial epidermis compared to field-cultivated plants ($P < 0.05$; Fig. 1). This agrees with previous reports in the Musaceae (reviewed by Carr, 2009; Sumardi and Wulandari, 2010; Sánchez-García et al., 2010): higher on abaxial epidermis for plants in the greenhouse than for plants in the field. In addition, different stomatal density was described between plants of the same genotype cultivated under different environmental conditions (e.g., light, vapour pressure deficit, and water availability) (Gay and Hurd, 1975; Reich, 1984; Camacho and Bellefleur, 1996). Bertolino et al. (2019) reviewed that the stomatal density and stomatal size may be adjusted depending on the prevalent conditions (e.g. greenhouse and controlled growth conditions) and that they have an impact on plant water use and carbon gain, reflecting in the less stressful environmental conditions for the greenhouse plants compared to those of the field.

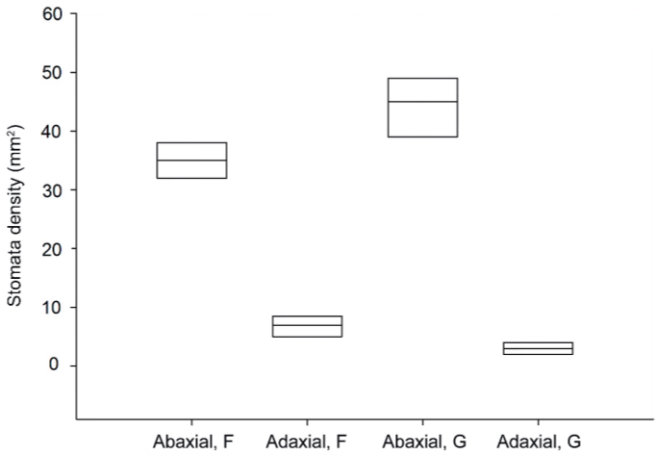

Fig. 1: Stomatal density of cultivated banana leaves (Grand Nain genotype). F = field; G = greenhouse. Statistical difference ($P < 0.05$) was determined by the Kruskal-Wallis test, n = 5.

In vitro physiological measurements of leaf fragments

Electron transport rate (ETR), and maximum quantum efficiency of photosystem II (F_v/F_m), and yield were higher in leaf fragments on benzimidazole-containing media than in those without benzimidazole during most measurements. A low variation of ETR was observed in leaf fragments with benzimidazole, compared to those without it, which showed an important reduction (11.65 and 4.02 µmol electrons m^{-2} s^{-1} from day 1 and 15 respectively; Fig. 2a) (F = 8.3, $P < 0.05$).

The present study results support the effectiveness of using leaf fragments under *in vitro* conditions to study the *Mycosphaerella-Musa* interaction. Leaf fragments without benzimidazole showed the lowest values of F_v/F_m (~0.1), which might indicate photodamage or photoinactivation of the protein D1 in photosystem II (Hou-Sung and Niyogi, 2008).The maximum difference of ETR between treatments was 9.54 µmol electrons m^{-2} s^{-1} on day 15. Also, F_v/F_m was higher in leaf fragments with benzimidazole than those without it.

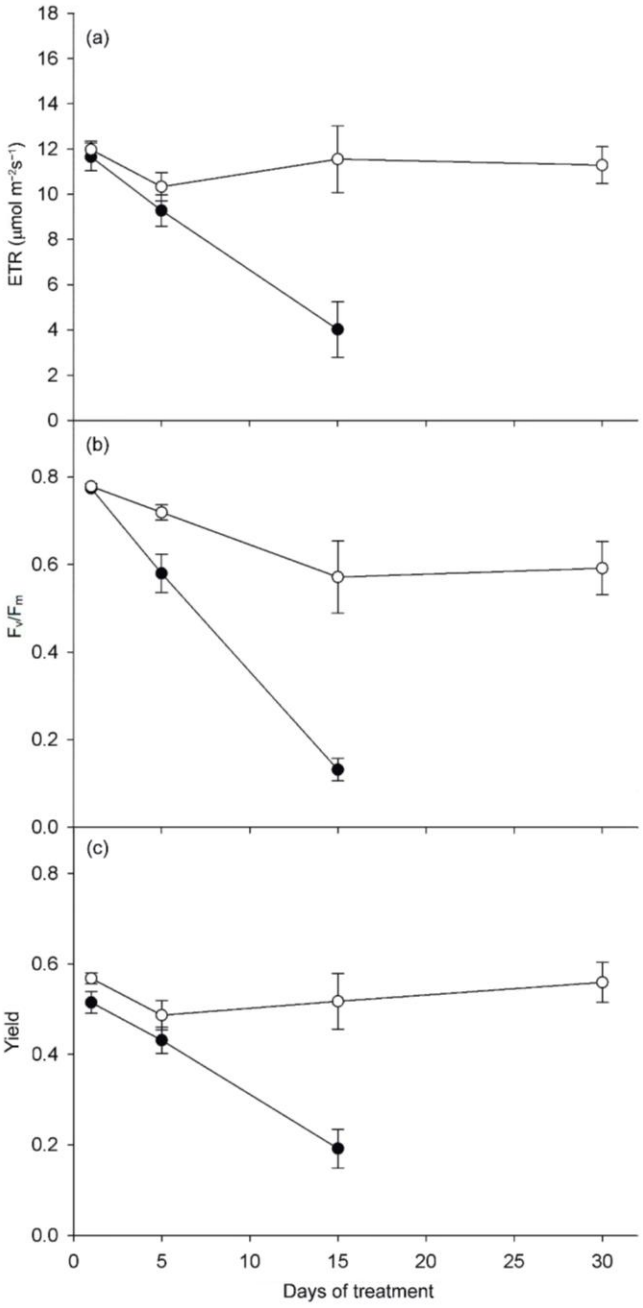

Fig. 2: Electron transport rate (a), maximum quantum efficiency of photosystem II (b), and yield (c) of leaf fragments of the Grand Nain banana genotype (control, *closed circles*, and benzimidazole treatment *open circles*). Data are means ± SE, n = 10. Statistical difference was determined by analysis of variance (ANOVA, $P = 0.05$) and the Fisher post hoc test, with exception of F_v/F_m of photosystem II, where the Mann-Whitney test was used.

The highest values were recorded at the beginning of the experiment, while the lowest value (0.1) was observed in leaf fragments without benzimidazole on day 15 (Fig. 2b). After 30 days, F_v/F_m decreased 25 % in fragments on medium with benzimidazole. Consistent with the ETR values, Yield was constant in leaf fragments on benzimidazole (Fig. 2c); control leaf fragments showed a reduction of 62 % from day 1 to 15 ($F = 7.368$, $P = 0.001$). Fig. 3 shows the leaf fragments colouration without (Fig. 3a) and with benzimidazole (Fig. 3b) in the medium after 30 days in *in vitro* culture. Since 60 years ago, it has been shown that benzimidazole, allows the maintenance of the green colour for detached leaves and their physiological functions, such as photosynthesis, RNA synthesis, and protein synthesis in other plant species (Person et al., 1957; Wang et al., 1961), and our results confirmed that the photosynthetic apparatus was preserved.

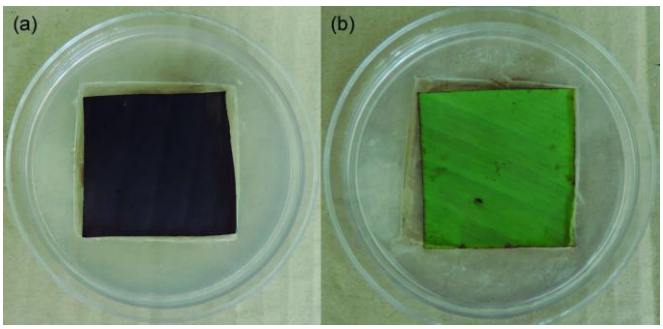

Fig. 3: Banana leaf fragment without benzimidazole (control; a) and with benzimidazole treatment (b) in the medium after 30 days in *in vitro* culture incubated at 26-30 °C, with a 12-h photoperiod and 72 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD).

Maximum photosynthesis (*A*) values were measured in leaf fragments with benzimidazole in the medium at day 5 (1.51 µmol CO_2 m^{-2} s^{-1}), and *A* was always higher for fragments with benzimidazole than those without it ($F = 4.365$, $P = 0.003$; Fig. 4a). Values of *A* in fragments with benzimidazole in the medium were similar at day 30 than at day 1; for fragments without benzimidazole in the medium, *A* could only been measured until day 15 (Fig. 4a). Transpiration rate (*E*) was higher for control leaf fragments in the dark compared to the other fragments (day 1; Fig. 4b); and *E* was similar between treatments at day 5, but at day 15 rates were significantly higher for leaf fragments with benzimidazole in the medium than for fragments without benzimidazole ($F = 9.3885$, $P < 0.05$). Thus, results obtained through this method could be comparable with information obtained directly from plants in the field. In addition, benzimidazole is cheap and easy to obtain from the supplier.

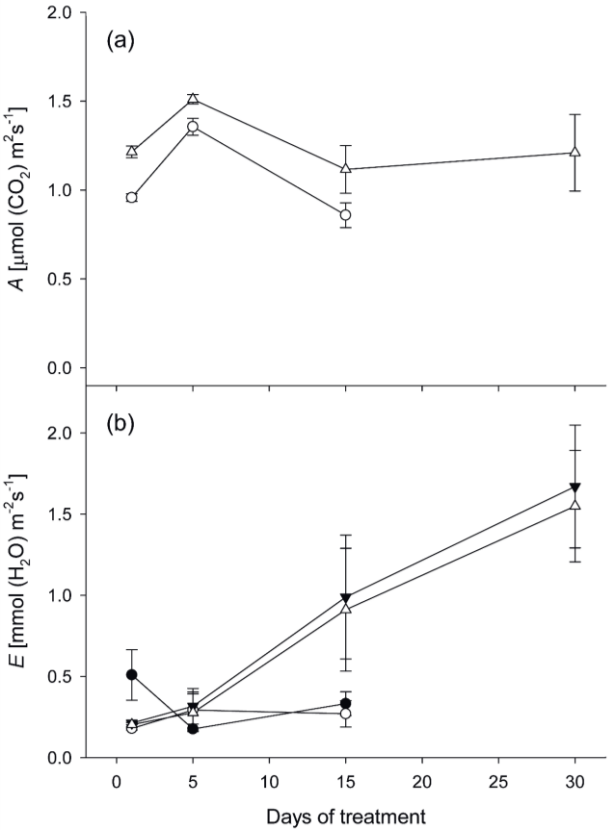

Fig. 4: Carbon dioxide uptake (a) and transpiration rate (b) of leaf fragments of the Grand Nain banana genotype in the dark (control, closed circles, and benzimidazole treatment, closed triangles) and in the light (control, open circles, and benzimidazole treatment, open triangles). Data are means ± SE, n = 10. Statistical difference was determined by analysis of variance (ANOVA, $P = 0.05$) and the Fisher post hoc test.

Arzanlou et al. (2007) reported large variations in *M. fijiensis*'s biomass in banana leaf fragments infected artificially. Inconsistency among replicates of artificially infected banana leaf fragments could arise from variation in germination and infection rates, since conidia germination is not synchronic *in vitro* (Peraza-Echeverría et al., 2008). Nevertheless, lesions are also asynchronous in the field (Rieux et al., 2014). Therefore, the use of *in vitro* infected banana leaf fragments for studying black Sigatoka disease should be not excluded. Although, the *in vitro* fragments have shown larger variation than in field samples in molecular analyse. This variation could be due to the criterion for sampling; for leaf fragment, it is after *in vitro* inoculation, while for field samples it is when the black Sigatoka symptoms appear since initial infection (time zero is unknown). Our results indicate that banana leaf fragments maintained their photosynthetic parameters under *in vitro* conditions for up to 30 days when benzimidazole was in the culture medium. In fact, it has been established that this is enough time for establishment and penetration of the fungus *M. fijiensis* via stomata into banana leaf fragments *in vitro* (Peraza-Echeverría et al., 2008), and it has recently been shown that this technique is appropriate to study gene expression in the *M. fijiensis-Musa acuminata* interaction (Rodríguez-García et al., 2016).

Physiological measurements in the greenhouse

At midday, in March, leaves had an ETR of 59 ± 4.1 µmol m^{-2} s^{-1} (Fig. 5a), and F_v/F_m was close to 0.8 at dawn with no differences in Yield during the day ($P > 0.05$; Fig. 5b), indicating that no environmental stress occurred in March (dry season) and in September (rainy season) (Björkman and Demmig, 1987). However, at the end of the day, Yield was higher in plants measured in September than those measured in March. Senevirathna et al. (2008) suggest that chlorophyll fluorescence indicates short-term, dynamic photoinhibition under high light conditions, and this could be related to the low light environment during measurements (~200 µmol $m^{-2}s^{-1}$), which was much lower than for plants in the field. Also, CO_2 uptake was higher in March than in September during most of the day (Fig. 5c), with maximum values at 9:00 h (4.29 ± 0.25 µmol CO_2 $m^{-2}s^{-1}$). In the afternoon of March, plant transpiration rates were significantly higher ($F = 33.55$, $P < 0.05$; Fig. 5d) than for plants in September; the highest value was 3.32 ± 0.16 mmol H_2O m^{-2} s^{-1}at 15:00 h. Leaves of Grand Nain plants received an average of 9 ± 0.9 mol m^{-2} d^{-1} PPFD during March, and 4.4 ± 0.4 mol m^{-2} d^{-1} PPFD in September.

Compared to leaf fragments under in *in vitro* conditions, greenhouse banana leaves had a higher ETR because they received more light, but leaves fragments showed high F_v/F_m values than leaves. On the other hand, leaves exhibited a yield of 0.22 ± 0.01 at midday, during the dry season, (Fig. 6b), and no yield recovery was observed at dusk (18 h). Light curve showed that leaves had 37 µmol m^{-2} s^{-1} ETR at the light saturation point for greenhouse plants in September (Fig 6a). Leaves from plants in the greenhouse showed higher ETR than that from leaf fragments under *in vitro* conditions, indicating the higher light growth environment for the greenhouse than for the growth room. According to the light curve, the Grain Nain leaves reached a maximum ETR of 37.5 µmol m^{-2} s^{-1} at 250 µmol m^{-2} s^{-1} PPFD, which was a low light saturation point, because plants in this

locations can receive more than 2000 µmol m^{-2} s^{-1} of PPFD during the dry season (Cervantes et al. 2005). Therefore, these results suggest that *M. acuminata* might tolerate low light conditions reaching high ETR.

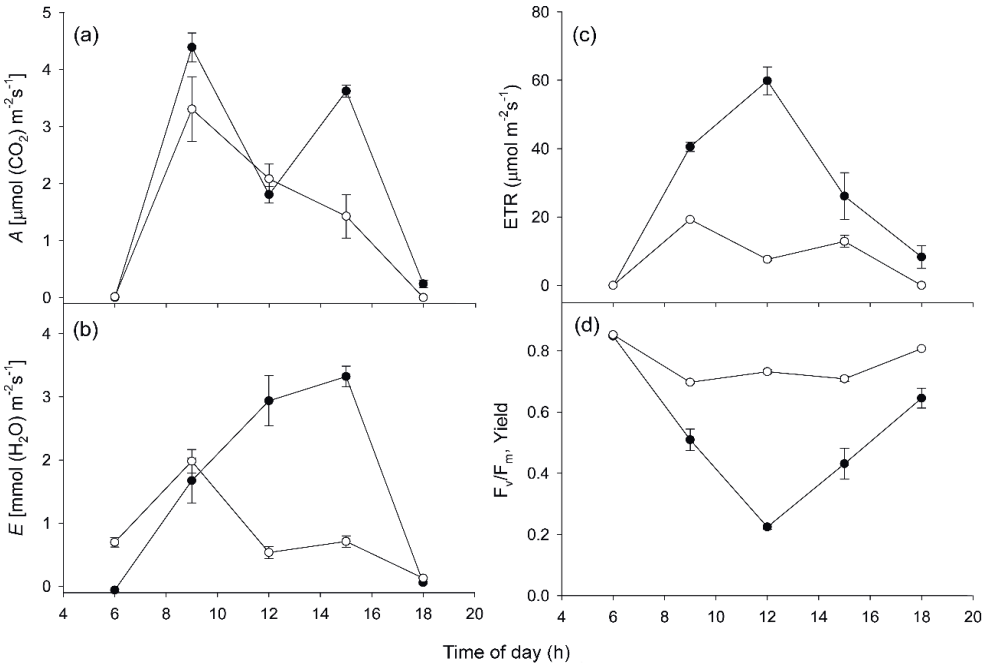

Fig. 5: Daily course of electron transport rate (a) and maximum quantum efficiency of PSII (6 h, dark), yield (day, light) (b), carbon dioxide uptake (c), transpiration rate (d) of the Grand Nain banana genotype on two dates (dry season, closed circle, and rainy season, open circles) under greenhouse conditions (n = 3). Statistical difference was determined by analysis of variance (ANOVA, $P = 0.05$) and the Fisher post hoc test.

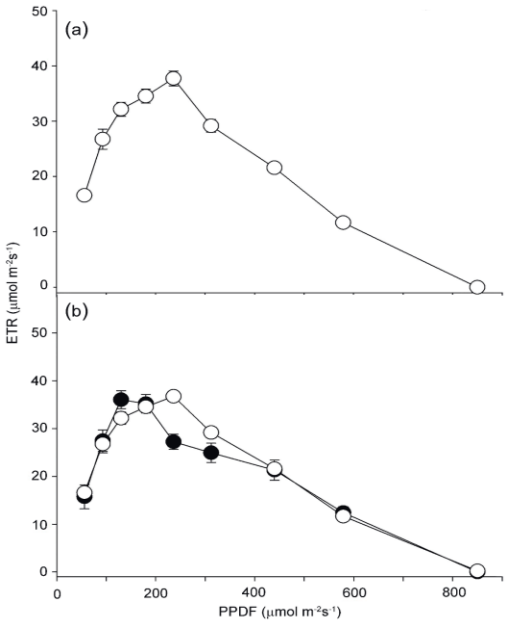

Fig. 6: Light-curve response of electron transport rate in leaves of the Grand Nain banana genotype under (a) greenhouse and (b) field conditions during dry (closed circles) and rainy (open circles) seasons. Error bars represent ± SE (n = 3).

Physiological measurements in the field

During the dry season, Gran Nain plants received 10 mol m^{-2} d^{-1} PPFD, and had an ETR of 29 ± 6 µmol electrons m^{-2} s^{-1} at midday (Fig. 7a); at this time leaf CO_2 uptake was significantly lower (3.16 ± 1.2 µmol CO_2 m^{-2} s^{-1}) than for plant leaves in the rainy season ($F = 6.641$, $P < 0.05$; Fig. 6c). In the dry season, a combination of high PPFD, and high temperatures may cause stomatal closure, and net photosynthesis is reduced (Turner and Thomas 1998). Accordingly, transpiration rates at midday were 6.0 ± 0.81 and 3.3 ± 0.55 mmol H_2O m^{-2} s^{-1} for the rainy and dry seasons, respectively ($F = 9.3533$, $P < 0.05$; Fig. 7d). During the rainy season, plants received 35 mol m^{-2} d^{-1} PPFD, and ETR increased significantly before midday, reaching a rate of 134 ± 6 µmol m^{-2} s^{-1} (Fig. 7a) at 09:00 h, and a maximum CO_2 uptake of 10.58 ± 0.8 µmol (CO_2) m^{-2} s^{-1} (Fig. 7c). Leaf F_v/F_m values of PSII did not differ between the dry and rainy seasons (Fig. 7b) in the Grand Nain leaves; the major difference in yield was at midday. During the rainy season leaves showed lower Yield values at noon (values were around 0.2), but they recovered at 18:00 h.

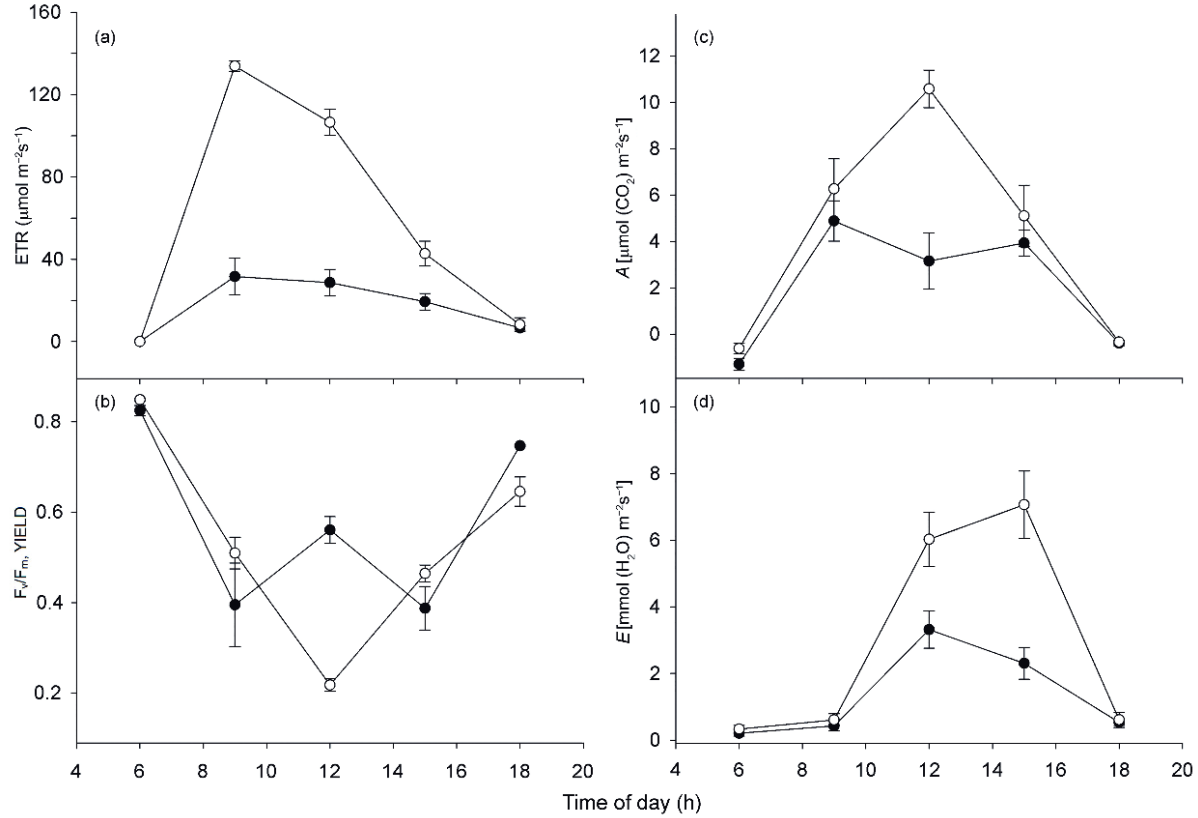

Fig. 7: Daily course of electron transport rate (a), maximum quantum efficiency of PSII, yield (during the day) (b), carbon dioxide uptake (c), and transpiration rate (d) of the Gran Nain banana genotype during two seasons (dry, closed circles, and rainy, open circles) under field conditions. Data were collected in the field (n = 3). Statistical difference was determined by analysis of variance (ANOVA, $P = 0.05$) and the Fisher post hoc test.

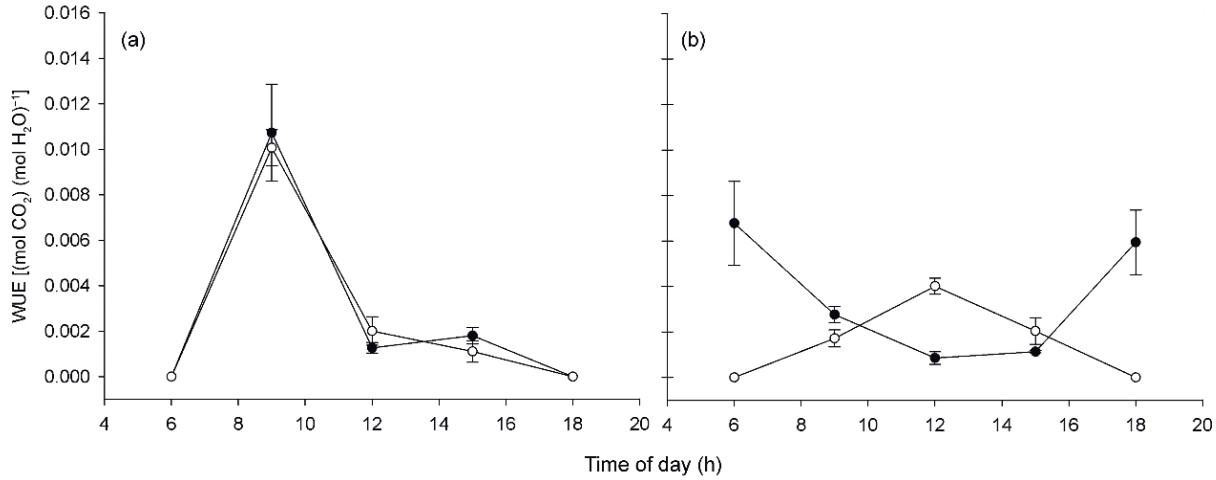

Fig. 8: Water use efficiency (WUE) in the Grand Nain banana genotype under field (a) and greenhouse (b) conditions in dry (closed circles) and rainy (open circles) seasons. Data are means ± SE (n = 3).

Plant leaves had similar water use efficiency (WUE) values during the dry and rainy seasons under field conditions (Fig. 8a), but in greenhouse conditions there were differences for the seasonal measurements during most of the day ($F = 15.619$, $P < 0.05$; Fig. 8b). Thomas et al. (1998) reported that high temperature, common in

dry periods, reduces net photosynthesis and increases leaf transpiration in leaves of *Musa* spp., and Eyland et al. (2021) that water conservation in these species is done at expense of potential carbon gain, due to stomatal closure to avoid water loss by transpiration. In this sense, the ability of Grand Nain plants to have a high WUE may confer a greater capacity to tolerate dry periods under field conditions since leaves were able to maintain similar CO_2 uptake during both the rainy and dry seasons (Fig. 8a).

Conclusions

In vitro leaf fragments with or without benzimidazole had differences in photosynthetic parameters, confirming that the *in vitro* banana-leaf-fragment technique preserves the photosynthetic apparatus and then it is suitable for studying the *M. fijiensis-M. acuminata* interaction. Under field conditions, leaves of the Grand Nain genotype exhibited differences in photosynthetic parameters between dry and rainy seasons; however, under greenhouse conditions, leaves showed lower values because of the low light environment.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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