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Natural ventilation and sucrose concentrations in the *in vitro* culture system affect the acclimatization of "Pérola" pineapple plants under different substrates

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Abstract

Plant tissue cultures represent a powerful set of tools that can be used in the production of pineapple seedlings. However, few studies have assessed the morphophysiological performance during the *ex vitro* acclimatization stage. Here, we evaluated the effect of different *in vitro* cultivation systems with sucrose reduction and types of flask sealing (without or with natural ventilation), as well as contrasting substrates on the growth and development of pineapple plants (*Ananas comosus* cv. Pérola) during *ex vitro* acclimatization. A randomized design in a 3 × 2 factorial was used with three *in vitro* cultivation systems [photomixotrophic (PM) + 30 g L^{-1} sucrose; photomixotrophic with natural ventilation (PNV) + 10 g L^{-1} sucrose; and PNV + 30 g L^{-1} sucrose)] and two *ex vitro* substrates (non-commercial and commercial). Our results showed that the commercial *ex vitro* substrate increased the height, stem diameter, and dry mass of aerial parts of plants by 25%, 14%, and 40%, respectively. Plants in PNV + 10 g L^{-1} sucrose and PNV + 30 g L^{-1} sucrose culture systems displayed reductions of 31% and 44% in respiratory rate, respectively. Notably, plants in a PNV + 10 g L^{-1} sucrose culture system displayed significant reductions of 36% and 41% for stomatal conductance to water vapor and transpiration rate, respectively. Thus, "Pérola" pineapple plants grown in a PNV + 10 g L^{-1} sucrose culture system using a commercial substrate exhibited improved gas exchange, with greater plant growth and development during acclimatization.

Keywords: *Ananas comosus*; flask sealing; photomixotrophic; photosynthesis; porous membranes.
Abbreviations: PA_photoautotrophic; PM_photomixotrophic; PNV_photomixotrophic with natural ventilation; PFV_ photomixotrophic with forced ventilation.

Introduction

Pineapple (*Ananas comosus* L. Merrill; family Bromeliaceae) – a fruit species endemic to the tropical regions of America, especially South America, where it has high commercial value – occupies a prominent international position as a fruit owing to the demand for fruits with high nutritional value, whether fresh or processed (Kessel-Domini et al., 2022; Gomez et al., 2022). It is anticipated that the global pineapple production can increase by 2.3% annually, reaching ~ 33 million tons in 2029 (Valdés-García et al., 2021). In Brazil, the leading global producer of pineapples, 64,787 ha of arable farmland was recorded, allowing for the harvesting of 1.7 billion fruits in 2020 (IBGE, 2022). However, to guarantee this yield, a high number of plants is required for planting on the available land (e.g., ~ 50,000 seedlings per ha) (Souza and Oliveira, 2021).

Currently, pineapples are mainly propagated in a vegetative manner through the sectioning of various plant parts, such as the shoots from the apex of the fruit, the peduncle, the region of insertion of the peduncle in the stem, axillary buds located in the leaf sheaths of the stem, and the buds of stem sections (Reinhardt and Cunha, 2006). The production of plants with a high level of heterogeneity, which leads to irregular production and, consequently, lower fruit quality, is one of the major problems facing these methods of propagation. Furthermore, plants of poor phytosanitary quality are produced, which promotes the spread of pests and diseases in the field, as exemplified by *Fusarium subglutinans* fungi, which can cause losses of 20% and 30% in seedlings and fruits, respectively (Oliveira-Cauduro et al., 2017; Miranda, 2021).

Driven by the demand for pineapple seedlings of a higher sanitary quality standard, plant tissue culture has become a powerful tool. Plant tissue culture, through a set of techniques, enables the commercial cloning of plants under aseptic cultivation conditions. In this sense, plant micropropagation can be subdivided into five stages: (i) plant stock immobilization and pre-treatment, and explant selection; (ii) culture establishment, elongation, and multiplication (e.g., heterotrophic, photomixotrophic, or photoautotrophic systems); (iii) rooting; (iv) weaning,

hardening, and acclimatization; and (v) transfer to the field (Soumare et al., 2021).

Plants in the conventional or photomixotrophic (PM) cultivation systems (i.e., sealed flasks and medium containing sucrose or another carbohydrate) are subjected to high relative humidity, greater accumulation of ethylene concentrations, and high sucrose supply. This leads to low gas exchange, CO_2 shortage during most of the photoperiod, and lower photosynthetic efficiency (Ashrafzadeh and Leung, 2021).

Although PM is the most often used cultivation system, morphogenetic processes can lead to the formation of leaves with mesophyll showing large intercellular spaces, a poorly developed vascular system, and the presence of non-functional stomata (Kozai and Kubota, 2001; Xiao et al., 2011; Martins et al., 2020; Fortini et al., 2021). Moreover, all these morphophysiological traits render the PM system harmful in seedling acclimatization, as evidenced by high mortality rates (Pinheiro et al., 2021).

The PM cultivation system can be optimized through the addition of natural ventilation (photomixotrophic with natural ventilation; PNV) or forced ventilation (photomixotrophic with forced ventilation; PFV). These systems reduce the morphophysiological disorders that commonly occur in PM because the use gas permeable films to optimize gas exchanges between the headspace of the flasks and external environment (Fortini et al., 2021; Louback et al., 2021). Under these conditions, it is possible to partially remove the carbohydrate source in the culture medium, but when the carbohydrate source is totally removed, a photoautotrophic (PA) system is obtained (Oliveira et al., 2021).

Although increasing attention has been paid to PNV, PFV, and PA crop culture systems as options to mitigate losses during acclimatization, this final step has often been neglected in several studies that have not examined plants under acclimatization conditions (Martins et al., 2020; Molinari et al., 2021; Oliveira and Aloufa, 2022). This step refers to the stage in which the *in vitro* plants are transferred to the *ex vitro* environment. Acclimatization is a limiting factor for most *in vitro* propagated species, owing to the great contrasts between *in vitro* and *ex vitro* conditions, and it is therefore necessary to research strategies that can be implemented at this stage in order to establish a successful *in vitro* culture system in all stages (Poniewozik et al., 2021).

In this regard, although understudied, the type and quality of the substrate can directly influence the acclimatization process, minimizing losses and favoring morphogenetic processes that lead to improved growth and development of plants in *ex vitro* conditions (Poniewozik et al., 2021; Pirata et al., 2022; Khandel et al., 2022). However, for economic reasons, many producers end up using the soil from their rural properties as substrate, which can have a negative impact on the quality of seedlings if no strategies are implemented to improve the physical, biological, and chemical properties of these soils.

The objective of this study was to: (i) evaluate the implications of different *in vitro* cultivation systems based on sucrose reduction and the use of porous membranes to seal flasks in subsequent *ex vitro* acclimatization stage; (ii) define the substrate that provides better photosynthetic performance, growth, and development of "Pérola" pineapple plants during the *ex vitro* acclimatization stage; and (iii) generate new information that can be used in the biotechnological propagation of this cultivar.

Results

Commercial substrate improves growth and biomass accumulation in the aerial part of pineapple plants during ex vitro acclimatization

Table 2 demonstrates that there were no significant interactions between *in vitro* culture conditions (PM, PNV + 10 g L^{-1} sucrose, and PNV + 30 g L^{-1} sucrose) and substrates (non-commercial and commercial) for all growth variables and dry mass ($P > 0.05$). In contrast, while analyzing the cultivation conditions and substrates independently, it was discovered that the types of substrates significantly affected the plant height, stem diameter, and dry mass of the aerial parts of these plants (Table 2).

In comparison to the measurements for the non-commercial substrate, the commercial substrate significantly increased plant height by 25% ($P < 0.01$; Figure 1A), stem diameter by 14% ($P < 0.05$; Figure 1B), and shoot dry mass by 40% ($P < 0.05$; Figure 1D). Although the number of leaves (10–20 units; Figure 1C) and total dry mass (0.46–1.49 g; Figure 1F) values of plants grown using the commercial substrate tended to be higher than those for plants grown using non-commercial substrate; the differences were not statistically significant ($P > 0.05$). However, for root dry mass (0.13–0.50 g; Figure 1E), the opposite was observed, with plants grown in non-commercial substrate displaying the greatest tendency towards high values, but no statistically significant increase ($P > 0.05$).

Photomyxotrophic cultivation with natural ventilation and ex vitro substrate types do not affect chlorophyll fluorescence parameters during ex vitro acclimatization of pineapple plants

Pineapple plants displayed significant interactions between *in vitro* cultivation conditions (PM, PNV + 10 g L^{-1} sucrose, and PNV + 30 g L^{-1} sucrose) and substrates (non-commercial and commercial) solely for the SPAD index ($P < 0.01$; Table 2). Regarding the other chlorophyll fluorescence variables (e.g., F_0, Fm, Fv/Fm, Rc/ABS, and PI), no significant interactions were observed between *in vitro* cultivation conditions and substrates ($P > 0.05$; Table 2), even when these factors were independently considered.

Plants cultivated *in vitro* under PNV displayed a significant decrease in the SPAD index (~ 17% less) (Figure 2A). Moreover, plants grown in commercial substrate displayed significant reductions of 12% (PNV + 10 g L^{-1} sucrose) and 18% (PNV + 30 g L^{-1} sucrose) in SPAD index compared to that grown in non-commercial substrate when considering the *in vitro* cultivation conditions (Figure 2A). Furthermore, excluding significant increases or decreases, initial fluorescence values ranged from 6,527–11,741 (Fig. 2B), maximum fluorescence values ranged from 33,540–54,025 (Fig. 2C), maximum quantum yield of photosystem II values ranged from 0.78–0.81 (Fig. 2D), energy absorbed per active reaction center values ranged from 1,208–2,132 (Fig 2E), and performance index values ranged from 2,332–5,376 (Fig 2D).

Photomyxotrophic cultivation with natural ventilation improves the gas exchange of pineapple plants during* ex vitro *acclimatization

Pineapple plants exhibited significant interactions between *in vitro* cultivation conditions (PM, PNV + 10 g L^{-1} sucrose,

and PNV + 30 g L^{-1} sucrose) and substrates (non-commercial and commercial) solely for respiration rate (inferred indirectly via photosynthetic response) ($P < 0.05$; Table 2). Notably, regardless of the treatments, pineapple plants did not show photosynthetic carbon assimilation when their gas exchange were analyzed (Figure 3A). In addition, analyzing the culture conditions and substrates independently revealed that the *in vitro* culture conditions had a significant effect on gas exchange (e.g., g_s, C_i, and E) ($P < 0.01$ and $P < 0.05$; Table 2).

Plants grown *in vitro* in PNV + 10 g L^{-1} sucrose and PNV + 30 g L^{-1} sucrose cultivation systems displayed significant reductions of 31% and 44% in respiratory rate, respectively, when compared to those grown in the PM + 30 g L^{-1} sucrose cultivation system (Figure 3A). In parallel, plants grown in the PNV + 10 g L^{-1} sucrose cultivation system displayed significant reductions of 36% (stomatal conductance to water vapor) and 41% (transpiration rate) compared to those grown in the PM + 30 g L^{-1} sucrose cultivation system (Figure B and D). Plants grown in the PNV + 10 g L-1 sucrose and PNV + 30 g L-1 sucrose cultivation systems displayed the same response pattern, both systems exhibiting a significant (approximately 35%) reduction in internal CO_2 concentrations compared to those concentrations in the PM + 30 g L^{-1} sucrose cultivation system (Figure C).

Discussion

Pineapple is widely cultivated in tropical and subtropical regions of the world, and is regarded as one of the most important tropical fruits in terms of global trade and economic interest (Dhurve et al., 2021; Kessel-Domini et al., 2022). In this context, plant tissue culture contributes to the rapid *in vitro* production of high quantities of pineapple seedlings with excellent physical, physiological, and sanitary qualities (Kessel-Domini et al., 2022). Here, the data showed that the use of commercial substrate led to gains in growth and plant biomass in the aerial part of the pineapple "Pérola" during the *ex vitro* acclimatization stage. Of note, based on gas exchange, the plants under *in vitro* culture system with natural ventilation and a reduced sucrose concentration (e.g., PNV + 10 g L^{-1} sucrose) showed better physiological performance than other culture systems.

Considering that the commercial substrate increased the growth in height, diameter, and dry mass accumulation in the aerial part of pineapple during *ex vitro* acclimatization, this was primarily owing to higher levels of macro-nutrients and micro-nutrients, and a larger cation exchange capacity in consonance with lower acidity levels in the commercial substrate compared to the non-commercial substrate (see Table 1). Several pineapple cultivars grown using substrates with high nutrient availability displays proportional growth and development responses (Hanafi et al., 2009; Sossa et al., 2019; Cunha et al., 2021), which validates our findings.

Although it is typical for plants grown using commercial substrate (i.e., higher physical, biological, and chemical quality) to exhibit substantial gains in plant growth and development, our results demonstrated the same dose-dependent pattern of response in the *ex vitro* acclimatization stage of pineapples. This is an under-studied, but crucial, step in the culture of plant tissues where it is important to avoid losses and to provide adequate conditions that ensures the best quality seedlings. Therefore, it is strongly recommended that high-quality substrates are used to counteract the detrimental effects of *in vitro* cultivation during the acclimatization stage (Santos and Smozinski, 2015; Amghar et al., 2021; Pirata et al., 2022).

In general, the balanced availability of nutrients in the soil represents one of the primary variables responsible for good agronomic performance of plants; however, this can vary based on the nutritional requirements intrinsic to various agricultural species (Mengel and Kirkby, 2001). In this study, the number of leaves did not increase during acclimatization, but there was a better response on morphogenic processes, leaf expansion, and gas exchange in leaves that had already been emitted during the *in vitro* cultivation period. This, in turn, led to increases in CO_2 fixation and, consequently, the accumulation of dry mass in plants grown using the commercial substrate.

Mineral nutrients have many functions in plants, such as being a structural component, an enzymatic constituent, or an activator or enzymatic cofactor. These roles are essential for positive responses to growth, physiology, and morphogenesis (Mengel and Kirkby, 2001). In contrast, the improvement in plant growth responses is not only because of the quality of the substrate, but also as a response to the photosynthetic performance that plays a role in CO_2 fixation, which is linked to many physiological processes in the entire plant (Yuan-Yuan et al., 2021). Furthermore, plants lacking well-developed leaf anatomical traits may experience photosynthetic limitations both *in vitro* and *ex vitro* even when grown using substrates with favorable physical and chemical properties (Copetta et al., 2021; Fortini et al., 2021).

In general, *in vitro* cultured plants exhibit morphological, anatomical, and physiological abnormalities (Martins et al., 2020; Fortini et al., 2021; Pinheiro et al., 2021). Notably, concerning photosynthetic performance, pineapple plants did not reveal potential obstacles in chlorophyll fluorescence parameters across all treatments, although there might have been limitations at the biochemical level in PM cultivation without natural ventilation (or conventional cultivation). Of note, PM cultivation with natural ventilation led to reductions in respiration, g_s, and internal CO_2 concentrations in plants, which were proportionally lower in *in vitro* cultivation with reduced sucrose concentration (e.g., sucrose 10 g L^{-1}) than in the other cultivation systems. Therefore, photosynthesis or respiration responses were unlikely to be associated with photochemical limitations in PM cultivation without natural ventilation; instead, the high respiration associated with the high g_s, and internal CO_2 concentrations observed in this culture system might have been because of diffusive dysfunctions and, mainly, obstacles in terms of biochemical reactions to CO_2 fixation.

The potential occurrence of CO_2 diffusion limitations in pineapple plants grown in PM culture systems without natural ventilation during the *ex vitro* acclimatization stage may be an inevitable consequence of specific anatomical characteristics in these conditions that favor the presence of mesophyll with large intercellular spaces, and a poorly developed vascular system (Kozai and Kubota, 2001; Xiao et al., 2011), although these leaf anatomy data were not accessed in this study. However, the introduction of natural ventilation and *in vitro* sucrose reduction rectified this phenotype as expected. Although we do not believe that the CO_2 diffusion limitation was the decisive factor that affected photosynthetic performance in the current study, these deleterious effects of *in vitro* histological differentiation

Table 1. Chemical analyses of non-commercial (Fazenda Escola de São Luís) and commercial substrate (Organic Compost Quixaba®).

Substrate	pH	OM	P	Sorption complexes (cmol/ dm³)						Complex saturation (%)			
	CaCl	(g/kg)	(mg/dm³)	K	Ca	Mg	H+Al	SB	CEC	V	Ca	Mg	K
Non-commercial	5.2	23.2	57.3	0.08	3.12	1.49	2.09	4.69	6.77	69.2	46.1	22.0	1.1
Commercial	6.3	48.8	150.8	0.73	5.11	3.28	0.45	9.12	9.57	95.33	53.4	34.3	7.6

pH: hydrogen potential; OM: organic matter; P: phosphorus; K: potassium; Ca: calcium; Mg: magnesium; H+Al: potential acidity; SB: sum of bases; CTC: cation-exchange capacity; V: base saturation.

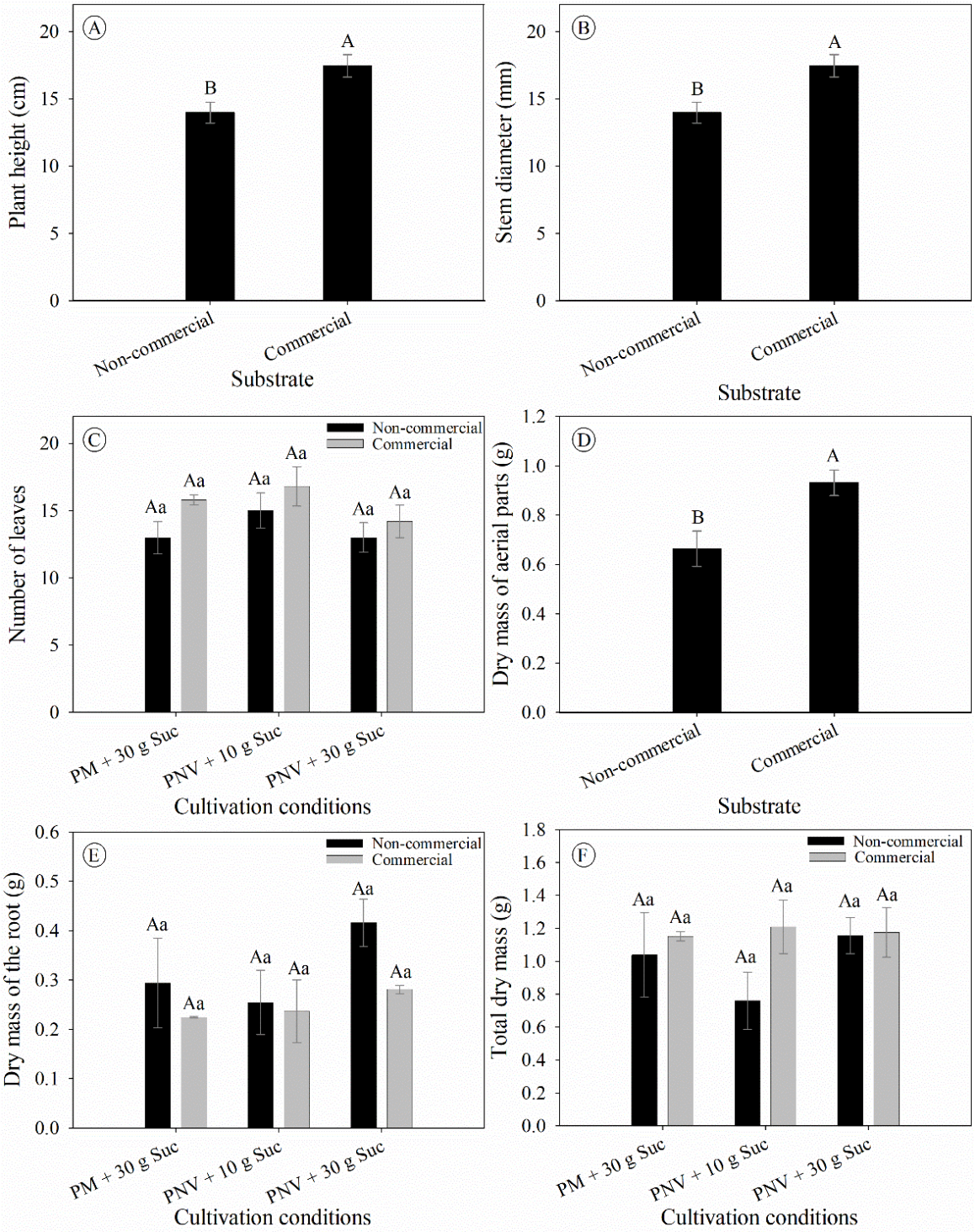

Figure 1. Growth parameters of "Pérola" pineapple plants under different *in vitro* cultivation conditions during the *ex vitro* acclimatization stage using two different substrate types. The parameters demonstrated by the different figures are as follows: A, plant height; B, stem diameter; C, number of leaves; D, dry mass of aerial parts; E, dry mass of the root; and F, total dry mass. Uppercase letters compare the use of substrates under the same cultivation conditions and lowercase letters compare the use of substrates under different cultivation conditions. The same uppercase or lowercase letters do not differ at 5% Scott-Knott's test. The bars represent the standard error (n = 5).

Table 2. F statistics and associated significance levels for the *in vitro* cultivation conditions (*CC*) and substrate (*S*) and its interaction (*CC* × *S*) on growth parameters and photosynthetic performance in "Pérola" pineapple plants.

Variables	Factors		
	Cultivation conditions (*CC*)	Substrate (*S*)	*CS* × *S*
Plant height	0.0643^{ns}	10.1627**	2.9825^{ns}
Stem diameter	1.1230^{ns}	6.5003*	0.0826^{ns}
Number of leaves	2.0399^{ns}	4.1945^{ns}	0.2444^{ns}
Dry mass of aerial parts	0.4248^{ns}	8.7408*	1.2116^{ns}
Dry mass of the root	1.9954^{ns}	2.6296^{ns}	0.5509^{ns}
Total dry mass	0.6323^{ns}	2.1283^{ns}	0.9685^{ns}
SPAD index	7.3056**	6.5203*	6.4343**
F_0	0.6609^{ns}	0.1983^{ns}	1.5199^{ns}
Fm	1.2517^{ns}	0.4337^{ns}	0.9300^{ns}
Fv/Fm	0.6103^{ns}	0.1677^{ns}	1.6386^{ns}
Rc/ABS	1.0384^{ns}	0.1703^{ns}	0.0308^{ns}
PI	0.7837^{ns}	0.3836^{ns}	0.0702^{ns}
A	35.4336**	0.4176^{ns}	4.2457*
g_s	6.0328*	0.1757^{ns}	1.0706^{ns}
Ci	11.8296**	1.3393^{ns}	3.3788^{ns}
E	8.3417**	0.0320^{ns}	1.0921^{ns}

ns: non-significant; level of significance $^{*}P < 0.05$ and $^{**}P < 0.01$

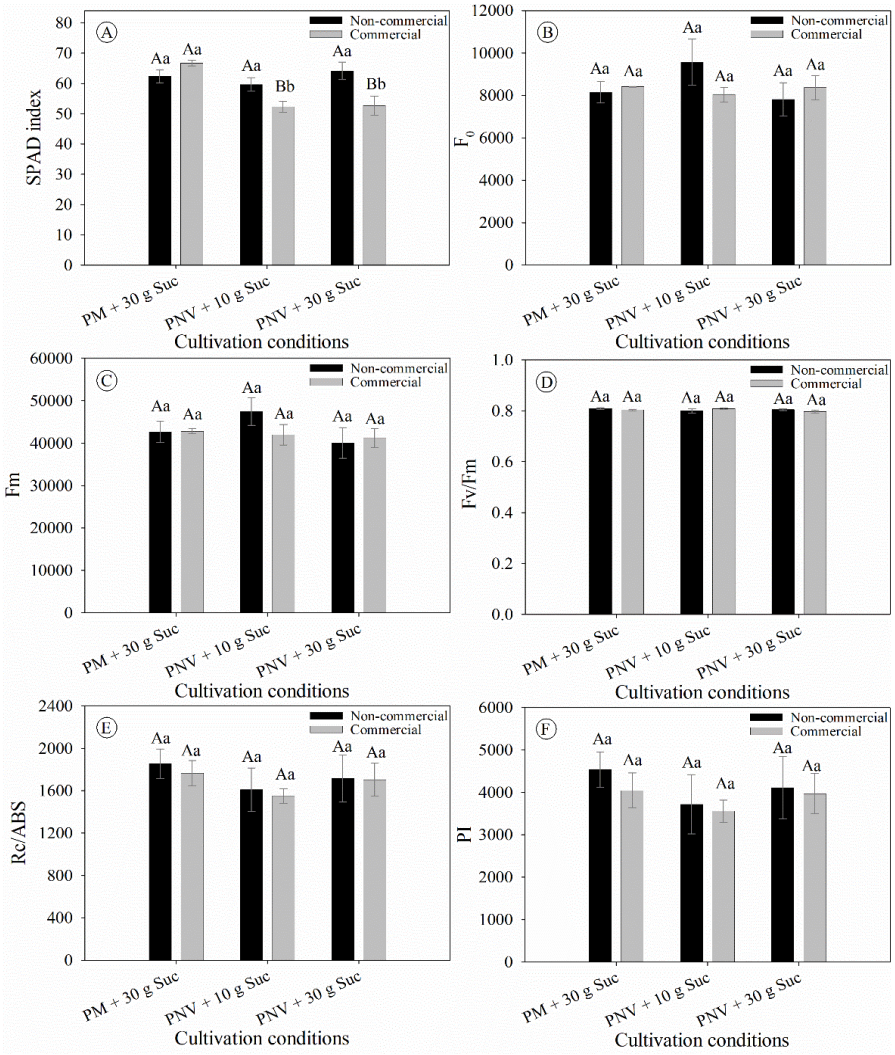

Figure 2. SPAD index and chlorophyll fluorescence parameters of "Pérola" pineapple plants grown under different *in vitro* cultivation conditions during the *ex vitro* acclimatization stage using two contrasting substrate types. The parameters demonstrated by the different figures are as follows: A, SPAD index; B, initial fluorescence (F_0); C, maximum fluorescence (Fm); D, maximum quantum yield of photosystem II (Fv/Fm); E, energy absorbed per active reaction center (RC/ABS); and F, Performance index (PI). Uppercase letters compare the use of substrates under the same cultivation conditions and lowercase letters compare the use of substrates under different cultivation conditions. The same uppercase or lowercase letters do not differ at 5% Scott-Knott's test. The bars represent the standard error (n = 5).

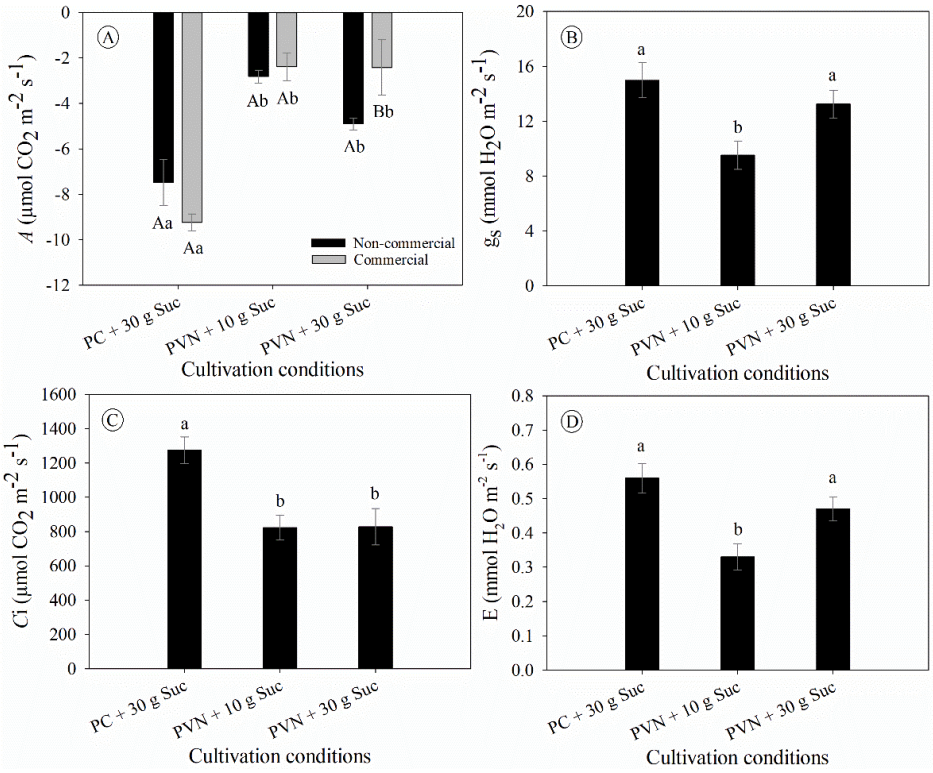

Figure 3. Leaf gas exchange parameters in "Pérola" pineapple plants under different *in vitro* cultivation conditions during the *ex vitro* acclimatization stage using two different substrate types. The parameters demonstrated by the different figures are as follows: A, net photosynthetic CO_2 assimilation (A); B, stomatal conductance to water vapor (g_s); C, internal CO_2 concentration (C_i); and D, transpiration rate (E). Uppercase letters compare the use of substrates under the same cultivation conditions and lowercase letters compare the use of substrates under different cultivation conditions. The same uppercase or lowercase letters do not differ at 5% Scott-Knott's test. The bars represent the standard error (n = 5).

cannot be ruled out even if they caused impairments on a smaller scale in *ex vitro* acclimatization (Martins et al., 2021). At the biochemical level of CO_2 fixation, the absence of porous membranes in the flask and higher sucrose concentrations during *in vitro* cultivation can inhibit the production of the key enzymes of photosynthesis, thereby contributing to the formation of a dysfunctional photosynthetic apparatus or being inhibited by saturation (Fortini et al., 2021). Indeed, under *in vitro* conditions, high concentrations of sucrose affect carbon assimilation in photosynthesis by inhibiting or reducing the activity of the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) (Desjardins et al., 1995; Lobo et al., 2015), and blocking the active carboxylation sites of Rubisco by phosphorylated sugars (Mayak et al., 1998). These potential *in vitro* responses may have been extended to the *ex vitro* acclimatization stage, as no new leaves had formed during the acclimatization stage when the data were examined.

In this study, the *E* reduction in "Pérola" pineapple plants grown in PM cultivation with lower sucrose concentration was verified. This demonstrated the improved functionality of the stomata and indicated that this cultivation system could produce plants that were better adapted to stress conditions, especially water deficiency. Previous studies have indicated that the thin leaf waxy layer and irregular stomatal cell function, particularly open stomata, may result in weak transpiration adjustments in plants that were grown *in vitro* without natural ventilation (Hazarika, 2006; Manokari et al., 2022). In addition, plants under these growing conditions have a low evapotranspiration rate, which reduces their ability to produce abscisic acid phytohormone and affects stomatal dynamics during acclimatization (Hasan et al., 2021).

The natural ventilation of flasks in conjunction with a reduced sucrose concentration in *in vitro* cultures provided a high gas exchange rate between the headspace and the external environment of the flask. This led to the formation of functional stomata similar to that observed in field conditions, allowing for greater control of transpiration and preventing the production of reactive oxygen species that damage cells owing to stress caused by the drastic change between *in vitro* and field conditions (Elyazid et al., 2021). In addition, the available carbon is mainly allocated to plant growth and development, rather than possible defensive strategies such as osmoregulation (Iqbal et al., 2022).

Collectively, the data showed that the use of natural ventilation and low sucrose mitigated the negative effects caused by the accumulation of ethylene gas in flasks without membranes (e.g., leaf senescence), increased the concentration of CO_2 inside the flask, and improved the physiological performance associated with gas exchange. In addition, it encouraged greater growth and development of the area part during the *ex vitro* acclimatization stage of the pineapple plants.

Materials and Methods

Plant material and culture conditions

Pineapple plants (*Ananas comosus* cv. "Pérola") used in the experiments were obtained from the *in vitro* Germplasm Bank at the Plant Tissue Culture Laboratory (Universidade Estadual do Maranhão, São Luís, MA, Brazil; 2° 31' 51'' S, 44° 18' 24'' W). These plants were cultivated in transparent glass

flasks (350 mL) containing 30 mL of MS culture medium (Sigma®, St. Louis, MO, USA; Murashige and Skoog, 1962). They were supplemented with 100 mg L^{-1} myo-inositol (Sigma®, St. Louis, MO, USA), 2.5 µM benzylaminopurine (Sigma®, St. Louis, MO, USA), and 30 g L^{-1} sucrose, and solidified with 7 g L^{-1} agar (AgarGel®, João Personal, PB, Brazil), and autoclaved at 121 °C and 108 kPa for 15 min. The plants were kept in a controlled growth room under an average temperature of 25 ± 2 °C, irradiance of 100 µmol m^{-2} s^{-1} (four tubular white LED lamps, T8, 9 W, Avant, São Paulo, SP, Brazil), and a 16-h photoperiod.

Explants (approximately 1.5 cm) were inoculated into clear glass flasks (350 mL) containing 50 mL of MS culture medium (Murashige and Skoog, 1962; Sigma®, St. Louis, MO, USA), supplemented with MS vitamins, 100 mg L^{-1} myo-inositol, 2.7 µM naphthaleneacetic acid, different concentrations of sucrose, and two bottle-sealing systems, resulting in different gas exchange rates (Batista et al., 2017): (i) photomixotrophic (PM) - polypropylene lids without membrane (14 µL L^{-1} s^{-1} CO_2 exchange rate) and culture medium with 30 g L^{-1} sucrose; (ii) photomixotrophic with natural ventilation (PNV) - polypropylene lids with two holes of 10 mm covered by two layers of microporous tape (25 µL L^{-1} s^{-1} CO_2 exchange rate) and culture medium with 10 g L^{-1} sucrose; and (iii) photomixotrophic with natural ventilation (PNV) - polypropylene lids with two holes of 10 mm covered by two layers of microporous tape (25 µL L^{-1} s^{-1} CO_2 exchange rate) and culture medium with 30 g L^{-1} sucrose, at an adjusted pH of 5.7 ± 0.1, solidified with 6.5 g L^{-1} agar (AgarGel®, João Personal, PB, Brazil), and autoclaved at 121 °C and 108 kPa for 15 min. The membranes used in the experiment were designed according to those described by Saldanha et al. (2012). The plants were kept in a controlled growth room under an average temperature of 25 ± 2 °C, 100 µmol m^{-2} s^{-1} of irradiance (four tubular white LED lamps, T8, 9 W, Avant, São Paulo, SP, Brazil), and a 16-h photoperiod.

***Ex vitro* acclimatization**

The culture medium was removed from the root systems of 60-day-old pineapple plants grown in PM, PNV + 10 g L^{-1} sucrose, and PNV + 30 g L^{-1} sucrose culture system by gently rinsing them under running water. The plants of each *in vitro* treatment were then transplanted into polystyrene pots (150 mL) containing two types of substrates, the first was a non-commercial substrate collected from the soil of Fazenda Escola de São Luís, São Luís, MA, Brazil (2° 35' 06.7" S, 44° 12' 30.9" W), and the second was a commercial substrate (Organic Compost Quixaba®, São Luís, MA, Brazil). The chemical properties of the two substrates were analyzed at the Terra Brasileira Agronomic Laboratory, Balsas, MA, Brazil (Table 1).

During the acclimatization stage, the plants were maintained under controlled conditions, which included an average temperature of 25 ± 2 °C, irradiance of 60 µmol m^{-2} s^{-1} (four tubular white LED lamps, T8, 9 W, Avant, São Paulo, SP, Brazil), and 16-h photoperiod. Plants were irrigated as required (e.g., dry substrate). At 60 d following the transplantation to *ex vitro* conditions, all growth and photosynthetic performance variables were assessed.

Plant growth parameters

The shoot height (H) was determined by measuring from the collar region of the seedling to the apical bud; the diameter (SD) was measured at the substrate level, at the collar region; and the number of leaves (NL) was determined by counting all leaves on the plant. In addition, shoots and roots were individually oven-dried at 70 °C until their mass remained constant, and their dry weight (DW) was accordingly obtained.

Chlorophyll estimation by Soil Plant Analysis Development readings

Chlorophyll estimates were obtained using a portable chlorophyll meter [Soil Plant Analysis Development (SPAD), model 502, Minolta Camera Co. Ltd., Japan] by accessing three points on the adaxial face of the third leaf counted from the apex to the base.

Gas exchange and chlorophyll a fluorescence in leaves

Gas exchange analyses were performed on the third completely grown leaf from the apex of plants using the open gas exchange system Li-6400XT (Li-Cor, Lincoln, NE, USA). The net photosynthetic CO_2 assimilation (A), stomatal conductance to water vapor (g_s), transpiration rate (E), and internal CO_2 concentration (C_i) were measured between 08:30 and 12:00 under an external CO_2 concentration of 500 µmol mol^{-1} air and an average temperature of 25 °C. To optimize stomata opening, all measurements were conducted under artificial and saturated light of 1500 µmol m^{-2} s^{-1} produced by a light-emitting diode generating 10% blue light.

Chlorophyll fluorescence measurements were performed on the same leaves used for the gas exchange assessments, and the following parameters were assessed: initial fluorescence (F_0), maximum fluorescence (Fm), maximum quantum yield of photosystem II (Fv/Fm), energy absorbed per active reaction center (RC/ABS), and the performance index (PI). The data were obtained using a portable non-modulated fluorimeter (Pocket-PEA, Hansatech, Norfolk, UK), which was calibrated to the leaf under dark conditions for 30 min using specific leaf clips for analysis in order to completely open the reaction centers with minimal heat loss and complete QA oxidation (Bolhar-Nordenkampf et al. 1989).

Statistical analyses

The design was completely randomized in a 3 × 2 factorial with five replications per treatment (three *in vitro* culture conditions: PM, PNV + 10 g L^{-1} sucrose, and PNV + 30 g L^{-1} sucrose; two *ex vitro* substrates: non-commercial and commercial); the experimental unit was one plant per pot. The data were statistically analyzed using one-way analysis of variance (ANOVA), and the means were compared using Scott-Knott's test with a significance level of 5%. All statistical analyses were performed using the software Genes (Cruz, 2013).

Conclusion

Our findings indicate that "Pérola" pineapple plants grown in natural ventilation systems in conjunction with 10 g L^{-1} sucrose in subsequent acclimatization using a substrate of higher physical, biological, and chemical qualities exhibited improved gas exchange, plant growth and development. In addition, these biotechnological strategies should be implemented in *in vitro* cultivation to produce higher-quality seedlings of this cultivar, directly contributing to the advancement of plant biotechnology and tropical agriculture.

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