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Effect of liquid media culture systems on yam plant growth (*Dioscorea alata* L. 'Pacala Duclos')

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The culture system type in liquid media influences yam plant growth ('Pacala Duclos' clone). In culture systems with forced renewal of internal atmosphere in culture flasks, Temporary Immersion System (TIS) and Constant Immersion System (CIS) with aeration through continuous bubbling in culture medium, higher results were obtained in morphological and physiological plant indicators in comparison with plants obtained in culture systems with passive renewal of internal atmosphere in culture flasks or Static Liquid System (SLS). In Temporary Immersion System, the best results were obtained after six weeks of culture in relation to total length (20.8 cm), axillary bud number (8.6), fresh weight (2.1 g) and dry weight (0.18 g) per plant, as well as photosynthetic pigment content (chlorophyll a, b, and total), net photosynthesis (15.3 μmol $C0_2 \cdot m^{-2} \cdot s^{-1}$), total transpiration (5.97 mmol $H_2O \cdot m^{-2} \cdot s^{-1}$), stomatal conductance (457 μmol $H_2O \cdot m^{-2} \cdot s^{-1}$) and leaf starch content (45.77 $mg \cdot gMF^{-1}$). Reducing sugar in culture medium with Temporary Immersion System was completely depleted, and mineral nutrients of lower contents (phosphorus, nitrogen, magnesium, calcium, iron, and manganese) in culture medium from this culture system could be related with plant growing. The results of this work could contribute to develop protocol for *in vitro* plant propagation of this yam clone.
Keywords. *Dioscorea alata*, *in vitro* culture, Temporary Immersion System, micropropagation, liquid medium.

Effet des systèmes de culture en milieu liquide sur la croissance des plants d'igname (*Dioscorea alata* L. 'Pacala Duclos'). Le type de système de culture en milieu liquide influence la croissance des plants d'igname (clone 'Pacala Duclos'). Dans des systèmes de culture avec renouvellement forcé de l'atmosphère interne des flacons de culture, le Système d'Immersion Temporaire (SIT) et le Système d'Immersion Constante (SIC), dont l'aération est assurée par la formation continue de bulles dans le milieu liquide, de meilleurs résultats furent observés au niveau des indicateurs morphologiques et physiologiques des plantes en comparaison avec des plantes provenant de systèmes de culture avec renouvellement passif de l'atmosphère interne en flacons de culture ou Système Liquide Statique (SLS). Dans le Système d'Immersion Temporaire, les meilleurs résultats furent obtenus après six semaines de culture et concernaient la longueur totale (20,8 cm), le nombre de bourgeons axillaires (8,6), le poids frais (2,1 g) et le poids sec (0,18 g) par plante, ainsi que le contenu en pigments photosynthétiques (chlorophylle a, b et totale), la photosynthèse nette (15,3 μmol $C0_2 \cdot m^{-2} \cdot s^{-1}$), la transpiration totale (5,97 mmol $H_2O \cdot m^{-2} \cdot s^{-1}$), la conductance stomatique (457 μmol $H_2O \cdot m^{-2} \cdot s^{-1}$) et le contenu en amidon des feuilles (45,77 $mg \cdot gMF^{-1}$). Le sucre réducteur dans le milieu de culture avec le Système d'Immersion Temporaire a été complètement épuisé, et les nutriments minéraux en moindre quantité (phosphore, azote, magnésium, calcium, fer et manganèse) dans le milieu de culture de ce système ont pu être mis en relation avec la croissance de la plante. Les résultats de ce travail pourraient contribuer au développement d'un protocole pour la propagation *in vitro* de ce clone d'igname.
Mots-clés. *Dioscorea alata*, culture *in vitro*, Système d'Immersion Temporaire, micropropagation, milieu liquide.

1. INTRODUCTION

Yam (*Dioscorea* spp.) crop has a wide range of uses in human life: staple food (as fresh consumption and processed products), animal food, and as raw material for industrial purposes (Tamiru et al., 2008). However extensive crop development in the principal yam producing countries of Central America and the Caribbean has been limited mainly because of lacking physiological, genetic, and healthy planting material.

In vitro plant propagation may help to overcome constraints related with the availability of high quality

planting material (Wheatley et al., 2003; Vaillant et al., 2005; Rodriguez, 2006). However almost all *in vitro* propagation protocols developed in yam up to now have shown erratic and low plant growth from nodal segments due to the use of semisolid culture media, conventional small-size culture flasks, and missing knowledge on the morpho-physiological development of *in vitro* plants (Malaurie et al., 1995; Medero et al., 1999; Chu et al., 2002; Borges et al., 2004; Balogun, 2009). So it has been necessary to look for alternatives with nodal segment explants in liquid media culture systems for plant growth, but little is known about the effect of these culture systems on *in vitro* plant physiology and morphogenesis (Cabrera et al., 2008).

This work was aimed at knowing the effect of liquid media culture system on yam plant growing (clone 'Pacala Duclos') and to determine in temporary immersion systems the nutrient depletion and preference in culture media, and to relate them with plant growth.

2. MATERIALS AND METHODS

2.1. Plant material

Plants of 'Pacala Duclos' (*Dioscorea alata* L.) clone were cultivated in a culture medium containing inorganic salts and vitamins as proposed by Murashige and Skoog (1962) (MS). Cistein (20 $mg \cdot l^{-1}$), sucrose (30 $g \cdot l^{-1}$) and agar-E at a final concentration of 5 $g \cdot l^{-1}$ were added to culture medium. Nodal segments showing axillary buds were obtained from *in vitro* plants in the third subculture.

The conditions of cultivation were a temperature of 25 ± 2°C and artificial illumination by means of white fluorescent (Sylvania, Daylight F40T12/D 40 W) lamps that provided an intensity of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The photoperiod corresponded to 16 h of light and 8 h of darkness.

2.2. Semi-automated culture systems

Temporary Immersion System (TIS). A setup of two 10 l flasks (Clearboys, Nalgene Company, USA), one serving as culture container and the other one as medium reservoir, was used. Flasks were connected with 6.0 mm silicone hoses passing through the cap down to the containers' bottoms. A flux of culture medium between the containers was controlled by two three-way electro valves. A programmable timer controlled immersion time and frequency. Hydrophobic filters (0.22 μm, MIDISART 2000, SARTORIUS Company) were fitted to each flask to ensure air sterility. Air pressure (1 $kg \cdot cm^{-2}$) from a compressor was regulated by a manometer. A 10-minute immersion time and an immersion frequency of 8 immersions per day were used.

Constant Immersion System with aeration through continuous bubbling in culture medium (CIS). This culture system consisted of a 10 l culture flask (Clearboys, Nalgene Company, USA) in which nodal segments and culture medium were placed. Air coming from a compressor flew through 6.0 mm silicone tubes down to the bottom of the container. Hydrophobic filters (0.22 μm, MIDISART 2000, SARTORIUS Company) were placed at the entrance and outlet of the culture flask to ensure air sterility. A compressor ensured a 25 $ml \cdot min^{-1}$ constant air flow.

Static Liquid culture System medium (SLS). A 10 l culture flask (Clearboys, Nalgene Company, USA) was used to grow nodal segments in a static liquid culture medium. Culture flasks were closed with caps, and hydrophobic filters (0.22 μm, MIDISART 2000, SARTORIUS Company) were fitted at the entrance and outlet of connectors to guarantee air sterility.

One hundred nodal segments were placed, each with an axillary bud, and a volume of 30 ml culture medium per nodal segment in each system of studied cultivation, for a total volume of 3 l culture medium. Experiments were repeated five times for each culture system.

2.3. Evaluation of morphological and physiological markers

After culturing for six weeks, 20 plants in each repetition per treatment were selected at random, and the following parameters were evaluated: total plant length (cm), axillary bud number per plant, and fresh (gFW) and dry weight (gDW) using an analytic balance (SARTORIUS). Before determining dry weight, plants were placed in a drying oven (SUTJESKA) at 70°C during 48 h. Besides the total plant number with hyperhydricity symptoms per replication was obtained in each culture system.

In order to determine the photosynthetic pigment content, 10 g of fresh leaves were taken. Three samples with three repetitions per treatment, thus a total of nine values, were considered. Chlorophylls a and b were extracted according to the protocol described by Porra (2002). Chlorophylls a, b, and total were counted following a procedure described by Lichtenthaler (1987).

In order to measure the photosynthetic, transpiration, and stomatal conductance activities, the youngest and completely expanded leaves from *in vitro* yam plants were used. Measurements were obtained with a CIRAS-2 (Portable Photosynthesis System, Europe, PP Systems, and UK) coupled to an

universal tray (PLC6) 4 h after starting the photoperiod treatment. The tray area was completely covered with the youngest and totally expanded leaves (2.5 cm^2). As yam plant is a C3 plant, a value of photo-synthetically active radiation (PAR) of 600 $W \cdot m^{-2}$ was chosen. Regarding carbon dioxide concentration and relative humidity, environmental values of 375 $\mu mol \cdot mol^{-1}$ and 90% were considered respectively. Measurements were carried out in five plants with 10 repetitions for a total of 50 values. In the case of photosynthesis, values were given in $\mu mol\ CO_2 \cdot m^{-2} \cdot s^{-1}$, transpiration in mmol $H_2O \cdot m^{-2} \cdot s^{-1}$, and the stomatal conductance in $\mu mol\ H_2O \cdot m^{-2} \cdot s^{-1}$.

To determine leaf starch concentration, 1 g of fresh weight was ground in liquid nitrogen using a mortar and a pestle. Three samples with three repetitions per treatment for a total of nine values were taken. Sugars were extracted in 5 ml ethanol at 80%. Extracts were centrifuged during 20 min at 10,000 g, 4°C to the vacuum in centrifuge Beckman (JB-21). Resultant pellets were resuspended in KOH 0.2 $mol \cdot l^{-1}$ promoting the alkaline hydrolysis during 12 h at 90°C. Later dissolving was carried out in sodium citrate with the β-amilo glycosidase (EC 3.2.1.3) (SIGMA) because of its enzymatic degradation according to a protocol described by Thomas et al. (1983). Starch was quantified using a calibration curve of potato starch (SIGMA). Values were expressed in $mg \cdot gMF^{-1}$.

2.4. Reducing sugar determination and mineral nutrients in culture medium in Temporary Immersion System

The responses from *in vitro* plant growth in temporary immersion systems were related with final reducing sugar contents and mineral nutrients in culture medium, and so, the following evaluations of each culture media were carried out: final reducing sugar concentration and mineral nutrient contents (N, P, K, Ca, Mg, Fe, Cu, Mn and Zn) at the beginning and at the end of plant growth.

To determine reducing sugars in culture medium, a protocol described in the manual of analytical techniques, ICIDCA (1974), was applied.

In relation to ion contents in culture medium, nitrogen (N) was determined with the Nessler´s calorimetric method, and phosphorus following the calorimetric method of ammonium metavanadate, according to the regulations from NRAG 564 (1982). Samples were read in a spectrophotometer Spekoll 11.

Potassium, calcium, magnesium, iron, copper, cobalt, and manganese were analyzed by dilution, based on the methodology of atomic-absorption spectrometry (Smith et al., 1972). Readings were taken in an atomic-absorption SP-9 Pye Unicam spectrometer.

The data relating to total plant length (cm), axillary bud number per plant, fresh weight (gFW) and dry weight (gDW) were statistically analyzed with a non-parametric test of Kruskall Wallis. Chlorophyll a, b, and total content means, photosynthetic activity, stomatal conductance, transpiration, and leaf starch concentration were compared with a simple variance analysis, and Turkey's test was applied.

3. RESULTS AND DISCUSSION

3.1. Evaluation of morphological and physiological markers

The type of cultivation system influenced significantly the growth of yam nodal segments. In the cultivation systems with forced renovation of the internal atmosphere of the cultivation (TIS and CIS) flask, superior results were obtained in the morphological indicators of the plants in comparison with those that were cultivated in the cultivation system with passive renovation of the internal atmosphere of the cultivation (SLS) flask. After six weeks of cultivation in TIS the highest values were obtained for total length, axillary bud number, fresh weight and dry weight per plant, and significant differences appeared as compared with plants growing in CIS and SLS (**Table 1**). The plants cultivated in SIT were characterized by an intense green coloration and featured expanded and well developed leaves (**Figure 1**).

Evaluating the different physiological variables made it possible to corroborate the development response of yam plants from TIS in comparison with plants cultivated in the other culture systems. In culture systems with forced renewal of internal culture flask atmosphere (TIS and CIS), results were better in physiological markers in comparison with plants grown in culture systems with passive renewal of internal culture flask atmosphere (SLS). The best results in relation to photosynthetic pigment content (chlorophyll a, b, and total), net photosynthesis, total transpiration, net photosynthesis relation and total transpiration, stomatal conductance, and leaf starch content were obtained after six weeks of culturing in TIS (**Table 2**).

The highest photosynthetic activity of plants from TIS and consequently the possibility of inducing greater photo-mixotrophic growth can be explained by the functioning of this culture system type. Escalona et al. (2007) pointed out that the periodic renovation of the internal atmosphere of the cultivation flask and the intermittent contact of the plants with culture medium in SIT resulted in the plants improving their relationship between photosynthesis and transpiration. These cultivation conditions allowed the

Table 1. Effect of culture systems on yam plant growing ('Pacala Duclos' clone) after 6 weeks of culture — *Effet des systèmes de culture sur la croissance du plant d'igname (clone 'Pacala Duclos') après six semaines de culture.*

Variables	Temporary Immersion System		Constant Immersion System		Static Liquid System	
	Mean	Mean ranges	Mean	Mean ranges	Mean	Mean ranges
Plant height (cm)	20.80	74.30[a]	18.60	46.70[b]	12.20	15.50[c]
Axillary bud number per plant	8.60	70.70[a]	7.80	50.30[b]	5.60	15.50[c]
Fresh weight (gFW) per plant	2.10	75.50[a]	1.85	45.50[b]	1.39	15.50[c]
Dry weight (gDW) per plant	0.18	75.50[a]	0.13	45.50[b]	0.08	15.50[c]
Plant number with hyperhydricity/culture system	3.40	15.50[c]	6.60	45.50[b]	9.40	75.50[a]

Mean ranges with non-common letters in the same line differ significantly by non-parametric tests of Kruskall Wallis for $p < 0.05$ (n = 100) and plants with hyperhydricity symptoms (n = 5) — *Les moyennes suivies de lettres différentes dans la même ligne diffèrent significativements selon les tests non-paramétriques de Kruskall Wallis pour p < 0,05 (n = 100) et pour les plantes avec des symptômes d'hyperhydricité (n = 5).*

Table 2. Physiological variables evaluated in yam plants (clone 'Pacala Duclos') growing in culture systems during six weeks — *Variables physiologiques évaluées dans les plants d'igname (clone 'Pacala Duclos') après six semaines de culture en fonction des systèmes.*

Variables	Temporary Immersion System	Constant Immersion System	Static Liquid System	Standard error ±
Chlorophyll a ($\mu g \cdot gFW^{-1}$)	46.22[a]	30.33[b]	15.66[c]	0.71
Chlorophyll b ($\mu g \cdot gFW^{-1}$)	29.77[a]	21.22[b]	10.66[c]	0.63
Chlorophyll total ($\mu g \cdot gFW^{-1}$)	75.99[a]	51.55[b]	26.32[c]	0.94
Net photosynthesis ($\mu mol\ C0_2 \cdot m^{-2} \cdot s^{-1}$)	15.30[a]	10.02[b]	4.10[c]	0.16
Total transpiration ($mmol\ H_2O \cdot m^{-2} \cdot s^{-1}$)	5.97[c]	6.76[b]	11.68[a]	0.07
Photo/Transpiration	2.56[a]	1.48[b]	0.35[c]	0.43
Stomatal conductance ($\mu mol\ H_2O \cdot m^{-2} \cdot s^{-1}$)	457.00[c]	518.00[b]	1 160.00[a]	9.32
Leaf Starch Content ($mg \cdot gFW^{-1}$)	45.77[a]	41.22[b]	24.22[c]	0.98

Means with non-common letters in the same line differ significantly by Turkey's test for $p < 0.05$ (n = 250), chlorophyll and starch concentration in leaves (n = 9) — *Les moyennes suivies de lettres différentes dans la même ligne diffèrent significativements selon le test de Turkey pour p < 0,05 (n = 250), pour la concentration en chlorophylle et en amidon dans les feuilles (n = 9).*

Figure 1. Cultivation flask with yam plants of the clone 'Pacala Duclos' at six weeks of cultivation, obtained in the temporary immersion system, starting from nodal segments — *Effet des systèmes de culture sur la croissance du plant d'igname (clone 'Pacala Duclos') après six semaines de culture.*

cultivated yam plants in TIS to present better stomatal functionality and relationship regarding the movement of gases with the entrance of CO_2 and the exit of water, which was reflected in a better relationship between photosynthesis and transpiration in comparison with the plants cultivated in the other cultivation systems.

According to Kozai et al. (2005) the culture conditions for photo-mixotrophic growing may induce chlorophyll synthesis in leaves from *in vitro* plants. Therefore the leaves of yam plants cultivated in TIS had a higher photosynthetic pigment content at the end of the growing stage compared with plants cultivated in any of the above-mentioned culture systems.

Aragon (2005) analyzed the chlorophyll content (a, b, and total) and its relation with photosynthesis during the rooting stage of plants from 'CEMSA ¾' clone in TIS under photo-mixotrophic conditions,

high illumination (250 μmol·m^{-2}·s^{-1}), and high CO_2 concentration (1,200 μmol·mol^{-1}). Plants with more chlorophyll a (63.54 μg·gMF^{-1}), b (35.67 μg·gMF^{-1}), and total (99.21 μg·gMF^{-1}) exhibited higher net photosynthesis values (2.41 μmol CO_2·m^{-2}·s^{-1}) and less total transpiration (1.53 mmol H_2O·m^{-2}·s^{-1}).

On the other hand, apple sprouts (*Malus domestica* Borkh.) were compared in continuous immersion bioreactors and in temporary immersion bioreactors by Chakrabarty et al. (2006). Sprouts cultivated in temporary immersion bioreactors showed higher chlorophyll a and b, fluorescence of chlorophyll, and net photosynthesis. Sprouts also presented higher fresh weight, dry weight, and length, and less hyperhydricidity.

Periodical renewal of the internal culture flask atmosphere in TIS and the intermittent contact of plants with culture medium made it possible to improve relations between photosynthesis and transpiration. These culture conditions caused yam plants cultivated in TIS to feature better stomatal conductance and a better relation according to gas movement with CO_2 entrance and water output, and this performance is reflected in a better relation between photosynthesis and transpiration in comparison with plants cultivated in other culture systems.

According to Afreen (2005) the lowest conductance values registered in the leaves of plants cultivated in TIS in comparison with leaf values of plants cultivated in CIS and SLS were a sign of better integrality in leaf structural development. This suggested that the mesophyll had more defined parenchyma palisade tissues, higher dimension in the intercellular spaces, higher chloroplast content, better epidermal cell development, and that epicuticular wax was present. Besides their stomas were more functional, as they would have permitted enough CO_2 to enter while water was retained as much as possible. The higher morphological development in leaves of plants cultivated in TIS made them more functional from the physiological point of view which was reflected in a better relation between net photosynthesis and total transpiration. The highest starch content in yam plant leaves was also observed in TIS. As is well known, the starch accumulated in leaves of *in vitro* plants may generate precursors due to the hydrolysis of sucrose in culture medium (Kozai et al., 2005) and to the carbonated skeletons formed through photosynthesis that may cause hexosas to become starch (Mendrano et al., 2001). In this case, the higher photosynthetic activity and the better functional mechanism to avoid unnecessary water losses in yam plants cultivated in TIS favored the highest accumulation of this compound with regard to other treatments. Researchers such as Aragon et al. (2009) determined that in plantain "CEMSA ¾" in TIS, the photomixotrophic growing conditions stimulated the carbohydrate accumulation in the starch content.

The culture conditions created in TIS resulted in the best plant growing in yam. According to Berthouly et al. (2005) and Escalona (2006), factors are combined to allow an intermittent contact of culture medium with plant material, so as to make possible an efficient nutrient contribution and a periodical renewal of the internal atmosphere in culture flasks. They have been able to increase plant growing and sprout proliferation in crops such as *Coffea arabica* L. and *Coffea canephora* L., pineapple (*Ananas comosus* L.), sugar cane (*Saccharum officinarum* L.), *Eucalyptus grandis* L., *Anthurium andraeanum* Schott, and in other Araceae species in comparison with protocols developed for these crops in culture media in semi-solid or static liquid state. The previous results permitted to explain why yam plants cultivated in TIS with higher chloropyll content (a, b, and total), higher photosynthetic activity, lower transpiration and stomatal conductance, as well as a better relation photosynthesis / transpiration, showed a better response on morphological indicators, such as total plant length, axillary bud number, fresh and dry weight per plant in comparison with plants growing in CIS and SLS.

3.2. Reducing sugar determination and mineral nutrients in culture medium in Temporary Immersion System

At the end of the 6-week plant growth period, the reducing sugars were completely depleted in the culture medium (**Table 3**). These results raise the question whether or not this compound turned into a limiting factor for plant development or influenced to a certain extent the photosynthetic activity recorded. Kozai et al. (2005) stated that plants taking exogenous carbohydrates from culture medium or endogenous carbohydrates produced by photosynthesis for growing showed a photomixotrophic metabolism, a process which could have occurred in yam plants cultivated in TIS.

Similar results in relation to photomixotrophic metabolism have been described in this culture system in crops such as pineapple by Escalona et al. (2003). Although sprouts were developed at high luminous intensity (225 μmol·m^{-2}·s^{-1}) and they carried out photosynthesis, *in vitro* plant growing depended more on reducing sugars and mineral nutrients from culture medium than on photosynthesis. However Aragon (2005) stated that *in vitro* plantain plants cultivated in temporary immersion bioreactors presented photomixotrophic growing, although not all the sugar in culture medium was depleted, and a high net photosynthesis (13.60 μmol CO_2·m^{-2}·s^{-1}) under

Table 3. Reducing sugar and mineral nutrient mean contents in culture medium at the beginning and at the end of growing period (six weeks of culture) of yam plants (clone 'Pacala Duclos') in temporary immersion system — *Contenu moyen en sucres réducteurs et en nutriments minéraux au début et à la fin de la période de croissance (six semaines de culture) des plants d'igname (clone 'Pacala Duclos') dans le Système d'Immersion Temporaire*.

Nutrient	Plant growing stage	
	Media initial ($mg \cdot l^{-1}$)	**Media final** ($mg \cdot l^{-1}$)
Reducing sugars	275.00	0.000
Mineral		
Nitrogen	1,700.00	270.000
Phosphorus	85.00	5.500
Potassium	845.00	555.000
Calcium	88.20	22.400
Magnesium	34.60	6.300
Manganese	4.35	2.120
Zinc	1.60	1.020
Copper	0.02	0.012
Iron	4.56	1.220

culture conditions of high light intensity and high CO_2 concentration were shown.

At the end of the growing period, the mineral nutrients with lower contents in culture medium in relation to initial values were: phosphorus, nitrogen, magnesium, calcium, iron, and manganese, while mineral nutrients such as potassium, copper, and zinc were maintained above 50% of the initial values (**Table 3**).

Different metabolic processes and functions in plant cells, where mineral nutrients of lower contents in culture medium are involved, may be related with the growth experimented by plants cultivated in TIS.

George et al. (2008) explained that phosphorus participates in the energetic metabolism and cell biosynthesis taking part in the glucose–6–phosphate and adenosinetriphosphate (ATP), in membrane stability, and is considered as an activator of adenosine diphosphate carboxylase enzyme, the most important enzyme involved in starch synthesis. This element also takes part in vital metabolic processes, such as photosynthesis and respiration. Nitrogen participates in the complex organic molecule synthesis, such as amino acids, proteins, and porphirine, which is a molecule from so important compounds since the metabolic point of view, as chlorophyll and in essential enzymes of the cytochrome group for photosynthesis and respiration. Besides the authors have also stated that calcium is involved in the structure and physiological properties of cellular membranes, and in the middle lamella of cell wall. Potassium takes part in the synthesis and metabolism of complex carbohydrates, because of its regulatory effect on several enzymes involved in carbon metabolism. Magnesium is also involved in the chlorophyll molecules of pigment systems I and II involved in photosynthesis.

Although Mn, Zn, Cu, and Fe mineral nutrients showed less variation in respect to initial concentrations in culture medium, Chajer et al. (2008) have also pointed out their roles as activators and regulatory cofactors of several enzymes that could have been involved in the growing of yam plants.

4. CONCLUSION

Liquid media culture systems influenced the growing of yam plants (clone 'Pacala Duclos'). Plants grown in TIS during six weeks showed the best results in the morphological indicators such as total length, axillary bud number, fresh weight and dry weight per plant, and in the physiological indicators evaluated, such as photosynthetic pigment content (a, b, and total chlorophyll), net photosynthesis, total transpiration, relation to net photosynthesis and total transpiration, stomatal conductance, and leaf starch content. Reducing sugars in culture medium of TIS were depleted, and the mineral nutrients with lower contents in culture medium in this culture system could be compared with plant growing.

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