REPORT

*Yam (*Dioscorea alata *L.) microtuber formation in Temporary Immersion System as planting material*

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ABSTRACT

Yam microtuber formation was developed in two culture stages. The type of culture system was defined and culture conditions were determined for microtuber formation, besides, the response of microtubers used as planting material in field conditions was evaluated. Evaluations of morphological and physiological indicators determined to use the temporary immersion system during plant growth and microtuber formation stages. With an immersion time of 15 minutes every six hours, in 60 mL culture medium per *in vitro* plant and four culture medium renewals, 355 microtubers with a fresh weight equal or higher than to 0.5 g per temporary immersion system were formed. Of these microtubers, 317 showed a fresh weight higher than 1.0 g and 121 of them presented a fresh weight equal or higher than 3.0 g. Microtubers were directly planted in field conditions, although those with a fresh weight equal or superior to 3.0 g presented the highest sprouting (91.30%) and survival (96.50%) percentages. Such plants showed the best responses in quantitative characters evaluated in the field. Starting from these results, a scheme for microtuber production in temporary immersion system was proposed, as well as the use of microtubers as direct planting material in field conditions.

Keywords: *in vitro* tuberization, semiautomatic culture systems, seeds

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RESUMEN

Formación de microtubérculos de ñame (*Discorea alata* L.) en sistema de inmersión temporal como material vegetal de plantación. La formación de microtubérculos de ñame se desarrolló en dos etapas de cultivo. Se definió el tipo de sistema de cultivo y se determinaron las condiciones de cultivo para la formación de microtubérculos, y además se evaluó la repuesta de los microtubérculos en campo que se emplearon como material vegetal de plantación. En la etapa de crecimiento de las plantas y en la de formación de microtubérculos se definió a través de la evaluación de indicadores morfológicos y fisiológicos emplear el sistema de inmersión temporal. Con 15 minutos de inmersión cada seis horas, un volumen de 60 mL de medio de cultivo por planta *in vitro* y cuatro renovaciones del medio de cultivo se formaron 355 microtubérculos con una masa fresca igual o superior a 0.5 g por sistema de inmersión temporal. De estos 317 presentaron una masa fresca superior a 1.0 g y 121 de ellos presentó una masa fresca igual o superior a 3.0 g. Fue posible plantar los microtubérculos directo en campo, aunque se determinó que aquellos con una masa fresca igual o superior a 3.0 g presentaron el mejor porcentaje de brotación (91.30%) y supervivencia de las plantas (96.50%). Estas mostraron las mejores respuestas en los caracteres cuantitativos que se evaluaron en campo. A partir de estos resultados se propuso un esquema para la formación de microtubérculos en sistema de inmersión temporal y su empleo como material vegetal de plantación directo a campo.

Palabras clave: tuberización *in vitro*, sistemas semiautomáticos de cultivo, semilla

*I*ntroduction

Yam (*Dioscorea* spp.) culture has contributed to energetic and nutritional requirements of a great part of the population in developing countries. Due to the wide range of uses and efficiency for digestible energetic production, yam will be considered as one of the most planted tuber for human food in the next years [1].

Nevertheless, their extensive development has been limited among other causes, due to the little availability of high quality planting material from the physiological and sanitary point of view. It mainly occurs because tubers that constitute the useful part of the plant for human consumption are used as planting material [2].

The microtubers production has a great potential as alternative for yam propagation [3, 4]. For such a reason, the main objective is aimed producing yam microtubers in temporary immersion system and to change culture conditions to increase the quantity and their fresh weight which would facilitate their use as direct planting material.

*M*aterials and methods

The investigation was carried out at the Research Institute of Tropical Root and Tuber Crops (INIVIT). Yam clone 'Pacala Duclos' (*Dioscorea alata* L.) from the Germosplasm Bank was used. Microtuberization was developed in two culture stages [5].

Effect of culture system type in liquid medium on *in vitro* tuberization

Temporary immersion system

The temporary immersion culture system (TIS) involved two 10.0 L culture flasks (type Clearboys (Nal-

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gene company, USA), one was used for nodal segment growth and the other as medium reservoir.

Constant immersion system with aeration through continuous bubbling in culture media

The constant immersion system (CIS) consisted of a 10.0 L culture flask (type Clearboys (Nalgene, USA) where nodal segments and culture media were placed. The air for bubbling came from a compressor.

Culture flask with static liquid culture medium with passive renewal of the internal atmosphere

The system, denoted as SLS, consisted of a 10.0 L culture flask (type Clearboys, Nalgene Company, USA) where nodal segments were placed in a static liquid culture medium (Control).

One hundred nodal segments with axillary bud and 30 mL culture medium volume per nodal segment were placed in each culture system studied. After having concluded the plant growth stage, a volume of 30 mL culture media tuberization per *in vitro* plant was added. The culture medium was not renewed during 18 weeks of culture.

This experiment was repeated seven times per each culture system, three repetitions were selected at random to evaluate morphological and physiological indicators during plant growth stage and the four remaining repetitions were used for the evaluations during the microtuber formation stage.

Evaluation of morphological and physiological indicators in the plant growth stage

After plant growing (six week culture), a total of 90 plants per culture system were selected at random and the following variables were evaluated: total plant length (cm), axillary bud number, fresh and dry weight (g) per plant. Besides, the total plant number with hiperhidricity was also counted.

Net photosynthesis, stomatal conductance and total transpiration were also evaluated. A total of 150 measures per culture system were carried out. For the determination of the photosynthetic (chlorophyll a, b and total) pigment content, 27 determinations per culture system were developed. Similar procedure was followed for starch content determination.

Evaluation of morphological and physiologic indicators in the microtuber formation stage

After concluding the microtubers formation stage (18 weeks of culture), a total of 120 microtubers were selected at random per culture system and the fresh weight (g), dry weight (g) and the diameter (mm) were measured.

Besides, the total microtuber number obtained from culture system, the microtuber number with a fresh weight equal or superior to 0.5 g and the number of hyperhydrated microtubers were assessed.

Determination of sugars reducers and mineral nutrients in the culture medium of the temporary immersion system

With the objective of relating the response on *in vitro* tuberization in temporary immersion system, the concentration of sugars reducers at the end of each culture stage was determined and at the beginning and the end of each stage, the mineral nutrient content (N, P, K, Ca, Mg, Fe, Cu, Mn and Zn) was determined

Effect of culture conditions in temporary immersion systems on microtuber formation

The effect of immersion times of 10.0 (Control), 15.0; 20.0 and 25.0 minutes were evaluated every three hours (eight immersions /day).

The effect of the immersion frequency each 3.0 (Control); 6.0; 12.0 and 24 hours were evaluated. The best previously obtained immersion time was used.

Effect of culture medium volume per *in vitro* cultivated plant: 15, 30 (Control), 60 and 90 mL culture medium volumes per *in vitro* cultivated plant were evaluated. The best previously obtained time and immersion frequency was used.

Each treatment contained 100 *in vitro* plants per TIS that had experienced a previous growing process of 6 weeks. 30 mL tuberization culture medium per *in vitro* plant was placed (except in the experiment where a survey on culture medium volume was carried out).

After finishing the microtuber formation stage (18 weeks of culture), the total microtuber number formed in TIS and the microtuber number with an equal fresh weight or superior to 0.5 g per TIS were quantified.

Effect of culture medium renewal, without renewal (Control), a culture medium renewal (nine weeks after culture), two culture medium renewals (six and 12 weeks of culture), three culture medium renewals (five, 10 and 15 weeks after culture), four culture medium renewals (four, eight, 12 and 16 weeks after culture) and five culture medium renewals (three, six, nine, 12 and 15 weeks of culture) were evaluated.

After microtuber formation stage (18 weeks of culture), the microtuber number obtained was classified and quantified according to fresh weight: 0.5-0.9 g; of 1.0-2.9 g; and equal and higher than 3.0 g.

Microtuber fresh weight influence on sprouting, survival and plant-morphoagronomical characters

Microtubers with 0.5-0.9 g fresh weight, microtubers with 1.0-2.9 g fresh weight and microtubers with a fresh weight equal or higher than 3.0 g, in vitro plants previously acclimatized with 30 cm stem length and at least four leaves and tuber crowns with 50 g fresh weight, conventional propagation (Control).

A randomized block design with five experimental plots per treatment was used. Experimental plots were formed by four mound rows with ten mounds per row, for a total of 40 mounds per plot and 200 mounds per treatment.

The experiment was planted in a carbonated calcium syalitic brown soil. Planting, irrigation and other cultural practices, as well as pest and disease control were carried out according to the Technical Instructive for yam crop.

Four weeks after culture in field conditions, sprouted microtuber number per plot was quantified and the percentage was determined. After six weeks of culture, survival percentage was recorded.

Ten plants from internal mounds were selected and the following variables were evaluated: Stem number per plant, stem length (m) after 24 weeks of planta-

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tion, tuber number / plant and tuber fresh weight (kg) /plant at harvest time.

Results and discussion

Effect of liquid culture system on *in vitro* tuberization. Evaluation of morphological and physiological indicators during plant growth stage

In culture systems with forced renewal of the internal atmosphere in the culture flask (TIS and CIS), higher results were obtained in total length (20.80 cm), axillary bud number (8.60), fresh (2.10 g) and dry (0.18 g) weight per plant with significant differences in relation to plants developed in CIS and in SLS.

The best results on photosynthetic pigment content (chlorophyll a, b, and totals), net photosynthesis, total transpiration, and relation on net photosynthesis and total transpiration, stomatal conductance and leaf starch content were obtained six weeks after culture in TIS.

The smallest conductance values in the leaves from plants cultivated in TIS, indicated according to [6], that these plants presented better integrality in the structural leaf development making them more functional from the physiologic point of view; so a better relation between net photosynthesis and total transpiration favored the biggest starch accumulation in the leaves.

Evaluation of morphological and physiological indicators in the microtuber stage formation

With the employment of TIS, microtubers of more quality reflected in the best results for the fresh, dry mass and diameter average were formed. These morphological indicators presented significant differences with regard to those that were formed in CIS and in SLS (Table 1).

After 18 weeks of culture, the highest total number of microtubers and microtubers with a fresh weight equal or higher than 0.5 g (241.80) was achieved, as well as the lowest number of hyperhidrated microtubers. In all cases, these results in TIS presented significant differences in relation to other culture systems. Microtubers with a fresh weight equal or higher than 0.5 g formed in TIS were distinguished for their quality, expressed in the highest dry weight and starch percentages. The possibility in TIS after each immersion to obtain a carbon dioxide concentration (CO_2) and oxygen (O_2) near to the atmospheric concentration and to eliminate the ethylene accumulation from the gassy space in culture flasks [7]. These culture conditions created in TIS allowed the highest microtuber formation in yam in comparison with those obtained in the rest of the culture systems.

Determination of sugar reducers and mineral nutrients in culture media in temporary immersion system

In the plant growth stage, after six weeks of culture, sugar reducers were completed exhausted in culture media. This result suggests that photomixotrophic nutrition could have happened in the case of yam plants cultivated in TIS.

At the end of the microtuber formation stage (18 weeks of culture), sugar reducers had been completely exhausted in culture media. The *in vitro* tuber formation occurs with high sugar levels in culture media [8] and as results of molecular genetic experiments, that sugars induce the expression of a wide gene variety and the specific protein synthesis of tuberization [9]. Sucrose has a double role in the microtuber development. Besides constituting a carbon source easily assimilable by *in vitro* plants for the conversion in starch, it is the most effective carbon source in the microtuber development. At the same time, sucrose at concentrations from 80 to 100 g/L provides a favorable osmorality for microtuber development [10].

When concluding the plant growth stage, the mineral nutrients with smaller content in the culture media regarding the initial value were: phosphorus (6.4%), nitrogen (15.8%), magnesium (18.2%), calcium (25.3%), iron (26.7%) and manganese (48.7%), while mineral nutrients as potassium, copper and zinc remained above 50% of the initial content. However, in the microtuber formation stage, after 18 weeks of culture in culture media, mineral nutrients as phosphorus were totally exhausted; nitrogen was at 8.8%, calcium at 18.6% and the potassium at 28.0% of the initial content in the culture media, while the mineral nutrients, magnesium, manganese, zinc, copper and iron remained above 50% of the initial content.

It is possible to relate the different metabolic processes and functions in plant cells, in which mineral nutrients of smaller content in culture medium are involved with the growth of plants cultivated in TIS; as well as in the stage of microtubers formation with the activation of the biochemical and physiologic mechanisms of *in vitro* tuberization.

Effect of culture conditions in the temporary immersion system on microtuber formation

Effect of immersion time: The best results were obtained with a 15 minute immersion time. After 18 weeks of culture with this immersion time, the highest total number of microtubers per TIS (309.40) and

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Table 1. Effect of the type of cultivation system in the stage of formation of microtubers of yam of the clone 'Pacala Duclos' to the 18 weeks of cultivation

	TIS[a]		TIS[a]		TIS[a]	
Variables/ microtubers	Mean	Range mean[d]	Mean	Range mean[d]	Mean	Range mean[d]
fresh weigh (g)	1.98	73.57[a]	1.45	40.50[b]	1.18	22.43[c]
dry weigh (g)	0.18	75.50[a]	0.10	39.03[b]	0.07	21.97[c]
Diameter (mm)	9.10	71.75[a]	7.50	37.40[b]	6.05	27.35[c]

[a]TIS: Temporary immersion system.
[b]CIS: Constant Immersion System with aeration through continuous bubbling in culture media.
[c]SLS: Culture flask with static liquid culture medium with passive renewal of the internal atmosphere
[d]Range means with non-common letters in oneself line differs according to non parametric test of Kruskall Wallis for $p < 0.05$ ($n = 120$).

the highest microtuber number with a fresh weight equal or higher than 0.5 g (300.41) with significant differences in relation to other immersion times were obtained [11].

Effect of the inmersion frequency: The best results were shown with an immersion every six hours. This immersion frequency resulted in the highest total number of microtubers per SIT (339.62) and the highest microtuber number with a fresh weight equal or higher than 0.5 g per SIT (328.81) after 18 weeks of culture. With this frequency, a gas composition renovation should have been achieved and that conforms the internal atmosphere of the culture flask and a nutrient availability in function of the requirements of *in vitro* cultivated plants favorable for *in vitro* tuberización, in comparison with immersion frequencies each three, twelve and twenty-four hours [11].

Effect of culture media volume for *in vitro* cultivated plant: The best results were obtained with 60 and 90 mL culture media volume per *in vitro* plant. After 18 weeks of culture, the highest number of microtubers were achieved per TIS (374.2 and 3710) as well as the highest microtuber number equal or higher than 0.5 g (365.0 and 3465). With these culture media volumes per plant, a higher number of axillary buds received the inductor stimuli of culture media for *in vitro* tuberización [11].

Effect of culture media renewal: Three times more microtubers with a fresh weight equal or higher than to 3.0 g were obtained with four culture media renewals in comparison with the treatment without renewal to culture medium.

Influences of microtuber fresh weight on sprouting, and survival and on morphoagronomic characters of plants derived from such microtubers

When microtubers with a fresh weight equal or higher than 3.0 g were planted directly in field conditions, the highest sprouting percentage (91.30%) among microtubers, without significant differences with the tuber crown were achieved.

The best survival percentages were obtained with plants coming from microtubers with a fresh weight equal or higher than 3.0 g (96.50%) without significant differences in plants coming from the tuber of the conventional propagation.

Evaluation of quantitative characters: In plants from all *in vitro* planting materials, 2.3 times more tubers than conventional propagation of plants coming from tuber crowns were obtained as average (Table 2).

This can be related to the effect of physiological and health renewal produced by *in vitro* culture. Microtubers have been considered as the best vegetable material to begin high quality "seed" production programs [12]. The present work for yam culture has corroborated these results, because a higher multiplication rate can be obtained in field conditions. The highest tuber number obtained in field conditions in plants from microtubers facilitates a planting material production programs and reduces the multiplication number that should be given to them in field conditions to satisfy demands of certified planting material.

Conclusions

Microtubers were formed in the three evaluated systems. The best results were obtained in temporary immersion system with 15 minutes immersion time every six hours, a 60 mL culture media volume per *in vitro* plant, and four culture media renewals. The microtuber number and fresh weight could be increased to allow using those with a fresh weight equal or higher than 3.0 g as planting material for direct plantation in field conditions.

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Table 2. Effect of the origin of the sections of tubers (100 g) in the answer of the plants of yam clone 'Pacala Duclos' (to the 36 weeks of cultivation) in the first vegetative (MV1) multiplication

Sections of tubers	Fresh mass of tubers (kg)/plant		Fresh mass of tubers (kg)/plant	
	Mean	Range mean[a]	Mean	Range mean
MV1 microtubers	3.80	64.50[a]	4.12	78.10[a]
MV1 *in vitro* plants propagated	3.60	56.50[a]	4.04	69.30[a]
Conventional propagation	1.60	10.50[a]	3.64	31.30[a]

[a]Range means with non- common letters in oneself column differs according to non parametric test of Kruskall Wallis for $p < 0.05$ (n =50).