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Culture media in the *in vitro* multiplication of 'Inhame da Costa' (*Dioscorea rotundada* Poir.)

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ABSTRACT: Yam plays an important socioeconomic role in Northeastern Brazil, but the use of low-quality seed tubers is an obstacle to cultivation. The micropropagation technique can work around this problem, as it allows the production of high quality phytosanitary seedlings. The objective of this work was to evaluate different culture media in the *in vitro* multiplication of 'Inhame da Costa' plants. Mini-cuttings with one lateral bud were inoculated in flasks containing MS, Galzy and 2GGC culture media, in solid and liquid consistencies, in an experimental design entirely randomized in a 3 × 2 factorial scheme. Cultivation was carried out in a growth room with 27 ± 1 °C, photon flow density of 30 µmol m^{-2} s^{-1} and photoperiod of 16 hours. At 90 days of cultivation, the plant height (cm), the number of shoots, the number of senescent leaves, the number of green leaves, the number of mini-cuttings and the fresh and dry masses of aerial parts and roots (mg). The explants were 100% responsive in all treatments and the 2GGC medium was the most efficient for *in vitro* multiplication, with liquid consistency being the most suitable for the micropropagation of 'Inhame da Costa'.

Key words: micropropagation; nutritional means; shoots

Meios de cultura na multiplicação *in vitro* do 'Inhame da Costa' (*Dioscorea rotundada* Poir.)

RESUMO: O inhame desempenha importante papel socioeconômico no Nordeste do Brasil, porém tem como entrave no cultivo a utilização de túberas-semente com baixa qualidade. A técnica da micropropagação pode contornar esse problema, pois possibilita a produção de mudas de alta qualidade fitossanitária. O objetivo desse trabalho foi avaliar diferentes meios de cultura na multiplicação *in vitro* de plantas do 'Inhame da Costa'. Miniestacas com uma gema lateral foram inoculadas em frascos contendo os meios de cultura MS, Galzy e 2GGC, nas consistências sólida e líquida, gerando um delineamento experimental inteiramente casualizado em esquema fatorial 3 × 2. O cultivo foi realizado em sala de crescimento com 27 ± 1 °C, densidade de fluxo de fótons de 30 µmol m^{-2} s^{-1} e fotoperíodo de 16 horas. Aos 90 dias de cultivo, foram avaliadas a altura de planta (cm), o número de brotos, o número de folhas senescentes, o número de folhas verdes, o número de miniestacas e as massas fresca e seca de parte aérea e de raízes (mg). Os explantes foram 100% responsivos em todos os tratamentos e o meio 2GGC foi o mais eficiente na multiplicação *in vitro*, sendo a consistência líquida a mais indicada para a micropropagação do 'Inhame da Costa'.

Palavras-chave: micropropagação; meios nutritivos; brotações

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Introduction

The yam is an herbaceous, climbing, tuberous plant belonging to the genus *Dioscorea*, which is the largest and most important within the family Dioscoreaceae. It has approximately 600 species, which are cultivated and consumed in various regions of the world, with its tubers constituting a substantial source of protein, lipids fiber, carbohydrates, vitamins, and minerals (Shajeela et al., 2011).

The species of commercial interest responsible for the largest yields of tubers are *Dioscorea cayennensis* Lam., *D. rotundata* Poir, *D. alata* L., *D. esculenta* (Lour.) Burkill, *D. bulbifera* L., and *D. dumetorum* (Kunth) Pax (Ouyabe et al., 2020). Among them, *D. rotundata*, variety 'Inhame da Costa', is the most widely cultivated, since it adapts easily to various soil and climate conditions and has greater acceptance by the population for consumption.

The yam crop is widely exploited in Brazil, especially in the Northeast region, where it presents itself as an agricultural alternative for expanding domestic and foreign markets, and can thus promote a source of income for family farming (Santos et al., 2016). However, technical and phytosanitary problems hinder the increase in production, such as the use of low quality seedlings that are a source of dissemination of pests and pathogens.

In this sense, biotechnology, through the micropropagation technique, has been contributing to the development of *in vitro* yam cultivation, promoting high multiplication rates (Nazir et al., 2022) and obtaining a propagative material with high genetic and phytosanitary quality through clonal cleaning (Ajayi et al., 2017). In this way, it prevents the spread of diseases and pests between generations, in a short space of time, and ensures the genetic stability of the material.

MS culture medium (Murashige & Skoog, 1962) is the most widely used for *in vitro* propagation of various *Dioscorea* species (Jirakiattikul et al., 2016; Nazir et al., 2021). However, the use of other culture media has also been studied (Ahanhanzo et al., 2010; Sêdami et al., 2017), aiming at the adequacy of a yam micropropagation protocol. Adjusting the composition of a basal culture medium is essential in controlling the morphogenetic responses of explants established *in vitro* (Kumar et al., 2017).

In this regard, Doukoure et al. (2000) developed and studied five culture media (2GH1, CL82-1, MHW78, 2GGC and M50) for the species *Dioscorea alata*, *D. esculenta* and *D. cayenensis-rotundata*. In this work, organogenesis was obtained in more than 50% of the genotypes grown in 2GGC and M50 culture media, which were then selected for *in vitro* cultivation of a yam collection. Birhan et al. (2021), tested different growth regulators for the protocol setup of *in vitro* propagation of a yam species (*D. alata*, bulcha variety) and found different concentrations of the regulators for the induction and multiplication phases.

As the yam crop is naturally recognized for its medicinal value and nutritional properties (Birhan et al., 2021), several works have sought the improvement of the micropropagation technique (Chukwunalu et al., 2018; Verde et al., 2021). However, there are still few studies that compare basic culture media in their *in vitro* cultivation. Thus, this work aimed to evaluate different nutrient media in the *in vitro* multiplication of 'Inhame da Costa' plants.

Materials and Methods

Mini-cuttings of approximately 1 cm in size, containing at least one lateral bud, were used from 'Inhame da Costa' plants previously cultivated *in vitro* in the Tissue Culture Laboratory of Embrapa Mandioca e Fruticultura, located in Cruz das Almas, Bahia, Brazil.

The mini-cuttings were inoculated, under aseptic conditions, in glass flasks containing MS (Murashige & Skoog, 1962), Galzy (Galzy & Compan, 1988) and 2GGC (Doukoure et al., 2000) culture media, whose compositions are shown in Table 1, in solid (30 mL of medium per flask) and liquid (15 mL of medium per flask) consistencies. All culture media were supplemented with 30 g L^{-1} sucrose and 1 g L^{-1} activated carbon, with the pH adjusted to 5.8 before autoclaving.

Table 1. Composition of MS (Murashige & Skoog, 1962), Galzy (Galzy & Compan, 1988) and 2GGC (Doukoure et al., 2000) culture media used for *in vitro* propagation of 'Inhame da Costa'.

Components (mg L^{-1})	Culture media		
	MS	Galzy	2GGC
Macronutrients			
NH_4NO_3	1,650.0	160.1	1,650.0
KNO_3	1,900.0	10.1	1,900.0
$CaCl_2.2H_2O$	440.0	-	440.0
$MgSO_4.7H_2O$	370.0	123.2	370.0
KH_2PO_4	170.0	122.5	170.0
$Ca(NO_3)_2.4H_2O$	-	495.4	-
$FeSO_4.7H_2O$	27.8	-	27.8
$Fe_2(SO_4)_3.5H2O$	-	18.4	-
$Na_2EDTA.2H_2O$	37.3	-	37.3
Micronutrients			
KI	0.830	0.249	0.830
H_3BO_3	6.200	0.025	6.200
$MnSO_4.4H_2O$	22.300	-	22.300
$MnSO_4.H_2O$	-	0.608	-
$ZnSO_4.7H_2O$	8.600	0.057	8.500
$Na_2MoO_4.2H_2O$	0.250	0.024	0.250
$CuSO_4.5H_2O$	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.043	0.025
Vitamins and aminoacid			
Thiamine-HCl (B1)	0.1	1	100
Pyridoxine-HCl (B6)	0.5	1	100
Nicotinic acid (B3)	0.5	1	-
Inositol (B complex)	100.0	10	10
Biotin (B7)	-	0.01	0.001
Calcium pantothenate (B5)	-	1	0.100
Ascorbic acid (C)	-	-	100
Glycine	2	-	200
Other supplements			
Activated charcoal	1,000	1,000	1,000
Saccharose	30,000	15,000	30,000
Agar	8,000	8,000	8,000

In addition, MS and 2GGC media were supplemented with 0.002 and 0.2 g L^{-1} glycine, respectively. The media with the solid consistency were solidified with 8 g L^{-1} agar, and in the liquid media the use of bridges was dispensed with. After inoculation, the mini-cuttings were grown in a growth room with 27 ± 1 °C temperature, photon flux density of 30 µmol m^{-2} s^{-1} and 16 hour photoperiod.

The experimental design was entirely randomized (DIC) in a 3 × 2 factorial scheme (culture medium × consistency), with five repetitions for the solid treatments and ten for the liquid ones. Each plot consisted of one flask with six mini-cuttings for the gelled media and three for the liquid media, totaling 30 plants per treatment.

At 90 days of cultivation, the following characteristics were evaluated: plant height (PH; in cm), number of shoots (NS), number of senescent leaves (NSL), number of green leaves (NGL), number of mini-cuttings (NMC), fresh mass of the aerial part (FMAP; in mg), root fresh mass (RFM; mg), dry mass of the aerial part (DMAP; mg), and root dry mass (RDM; mg).

The data obtained were submitted to the F test of variance analysis. The variables number of shoots, number of senescent leaves, number of green leaves, and number of mini-cuttings were transformed to √(x + 1) in order to meet the assumptions of the analysis of variance. The averages of the culture media were compared by the Tukey test and the averages of the consistencies by the F test, both at 5% significance level. Statistical analyses were performed with the aid of the R statistical program (R Core Team, 2019), using the ExpDes.pt package (Ferreira et al., 2018).

Results and Discussion

Under the experimental conditions analyzed, the explants were 100% responsive. It could be observed that in the variable number of shoots (NB) there was no significance, either for the isolated factors or for the interaction medium × consistency. The variables plant height (PH), number of green leaves (NGL), fresh mass of the aerial part (FMAP), root fresh mass (RFM), and root dry mass (RDM) were significant for the isolated factors and their interaction. However, for number of senescent leaves (NSL), number of mini-cuttings (NMC) and dry mass of the aerial part (DMAP) there was significance only for the isolated factors (Table 2).

Regarding the coefficients of variation (CVs), the lowest value was 10.67%, for NS, and the highest, 92.04 and 92.21%, for RFM and RDM, respectively, since they are interdependent values.

Ahanhanzo et al. (2010), working on *in vitro* culture of *Dioscorea cayenensis-rotundata*, found CVs ranging from 36.46 to 50.19%, while Simões et al. (2014), observed CVs between 29.42 and 47.42% in micropropagation of *Dioscorea rotundata*. Birhan et al. (2021), in turn, found a CV of 6.94% for plant height when working with the species *Dioscorea alata*.

In this experiment, the addition of the source of variation liquid medium may have contributed to the significant increase in CV, above 92.21%, since this condition was not studied in the previously cited works. Cardoso et al. (2018), conducting a study on the micropropagation of cassava varieties, obtained CV's between 6.52 and 54.43%. According to Carvalho (2013), tissue culture experiments usually show very high CV's, demonstrating that, despite the control of environmental conditions of cultivation, the distribution of response data does not usually follow the assumptions of normality, often requiring data transformation to perform the analysis of variance.

For all variables analyzed, MS, Galzy, and 2GGC media did not differ statistically from each other in solid consistency. However, there was a statistical difference in the liquid consistency, where Galzy showed lower averages compared to the other media, which can be attributed to its low availability and/or concentration of macro and micronutrients, reflecting in low plant metabolism, compared to MS and 2GGC. In this consistency, the MS and 2GGC media did not differ statistically from each other, with the exception of the NGL variable, where 2GGC presented the highest average (7.37) and differed statistically from the other nutrient media (Table 3). It is interesting to note that these two media have the same concentrations of macro- and micronutrients.

In vitro cultivation in liquid medium, nutrients and vitamins are more available and consequently tend to be better absorbed (Kuria et al., 2008), providing a better response in fresh mass production, which significantly contributed to better results involving this treatment compared to the gelled medium. These authors, working with potato (*Solanum tuberosum* L.), found higher biomass contents when applying the treatment involving liquid culture medium.

Table 2. Analysis of variance for the following variables: plant height (PH; cm), number of shoots (NS), number of senescent leaves (NSL), number of green leaves (NGL), number of mini-cuttings (NMC), fresh mass of the aerial part (FMAP; mg), root fresh mass (RFM; mg), dry mass of the aerial part (DMAP; mg) and root dry mass (RDM; mg) as a function of culture media and consistency in *in vitro* culture of 'Inhame da Costa' (*Dioscorea rotundata* Poir).

SV	DF	PH	NS	NSL	NGL	NMC	FMAP	RFM	DMAP	RDM
Medium (M)	2	50.69**	0.07^{ns}	0.26*	2.53**	1.46**	737109.00**	64087.00**	2348.90*	462.22**
Consistency (C)	1	18.87*	0.00^{ns}	1.42**	1.95**	1.22**	536694.00**	132250.00**	10671.10**	871.11**
M × C	2	26.35**	0.02^{ns}	0.02^{ns}	0.48*	0.27^{ns}	264468.00**	48463.00*	1337.80^{ns}	514.44**
Error		2.63	0.02	0.07	0.12	0.08	24041.00	9399.00	533.30	67.18
Average		6.30	1.48	0.71	5.82	3.94	441.78	105.33	51.11	8.89
CV (%)		25.73	10.67	24.48	13.84	13.94	35.10	92.04	45.18	92.21

** and * significant at 1% and 5% probability, respectively, by the F test. ^{ns} not significant at 5% probability.

Table 3. Average values of plant height (PH; cm), number of green leaves (NGL), fresh mass of the aerial part (FMAP; mg), root fresh mass (RFM; mg) and root dry mass (RDM; mg) of 'Inhame da Costa' (*Dioscorea rotundata* Poir) plants grown *in vitro* on different culture media.

Medium	Consistency	
	Solid	Liquid
	PH (cm)	
MS	5.25 aB	8.10 aA
Galzy	5.43 aA	3.56 bB
2GGC	5.46 aB	8.60 aA
	NGL	
MS	7.15 aA	5.42 bA
Galzy	6.28 aA	2.65 cB
2GGC	8.11 aA	7.37 aA
	FMAP (mg)	
MS	322.00 aB	636.00 aA
Galzy	244.00 aA	162.00 bA
2GGC	296.00 aB	759.00 aA
	RFM (mg)	
MS	26.00 aB	226.00 aA
Galzy	48.00 aA	25.00 bA
2GGC	12.00 aB	180.00 aA
	RDM (mg)	
MS	2.00 aB	19.00 aA
Galzy	6.00 aA	1.00 bA
2GGC	0.00 aB	16.00 aA

Averages followed by the same lower case letters in the columns and capital letters in the rows are not statistically different from each other by Tukey test at 5% significance level.

Comparing the results achieved between gelled and liquid media, it can be seen that MS and 2GGC media presented the highest averages for the variables plant height, above ground fresh mass, and fresh and dry mass of roots in liquid consistency (Table 3). However, for number of green leaves there was no difference between the consistencies of these media. Regarding the Galzy culture medium, the highest averages for plant height and number of green leaves were obtained in the solid consistency. However, the results obtained on this medium for aboveground fresh mass and root fresh and dry masses showed no statistical difference between the solid and liquid states.

In evaluation of explants of *Typhonium flagelliforme* L. in different culture media in liquid and solid consistency, Rezali et al. (2017) observed that the use of liquid culture media provided significant increase in the multiplication rate of plants.

For the number of senescent leaves and dry mass of the aerial part, the MS and 2GGC mediums did not differ statistically and presented the highest averages, 0.86 and 0.92, and 58.67 and 58.00 mg, respectively (Table 4), while the gelled Galzy medium provided, due to lower nutrient availability, greater root development to make up for this deficiency (Table 3). Consequently, this treatment generated a lower cellular metabolism and with it the lowest average for NSL (Table 4).

Regarding the number of shoots, there was no statistical difference between the studied media, with the highest average found in 2GGC (1.69). This same medium provided the highest average in the variable number of mini-cuttings (5.19), which was statistically superior to the averages provided by the other two nutrient media, respectively 3.97 and 2.67, for MS and Galzy.

Table 4. Average values of number of senescent leaves (NSL), number of shoots (NS), number of mini-cuttings (NMC) and dry mass of the aerial part (DMAP; mg) of 'Inhame da Costa' (*Dioscorea rotundata* Poir) plants grown *in vitro* on different nutrient media.

Medium	NSL	NS	NMC	DMAP
MS	0.86 ab	1.45 a	3.97 b	58.67 a
Galzy	0.37 b	1.31 a	2.67 c	36.67 b
2GGC	0.92 a	1.69 a	5.19 a	58.00 a

Averages followed by the same letter in the column do not differ by Tukey test at 5% probability.

In work with the species *Dioscorea deltoidea*, Nazir et al. (2021) found MS, supplemented with cytokinins and auxins, to be the ideal medium for *in vitro* multiplication of regenerated shoots. Doukoure et al. (2000), studying the behavior of *Dioscorea alata*, *D. esculenta* and *D. cayenensis-rotundata* species on different culture media, observed that the micropropagated plants developed and grew vigorously on 2GGC, which produced the highest averages for the shoot emission variable. This nutrient medium is the richest in organic nutrient sources among the analyzed media, so there is no need for the plants to metabolize inorganic nutrients into organic sources. In this way, energy needs to be spent on the synthesis, providing a more efficient reaction and sprout development.

Considering the consistency (Table 5), the liquid medium for number of senescent leaves and dry mass of the aerial part presented the highest averages (0.99 and 62 mg, respectively), differing statistically from the solid condition. However, the solid medium provided a higher average for number of minestrings (4.85) and statistically superior to the liquid medium. The averages for the number of shoots were very similar, 1.47 and 1.49, respectively, for the solid and liquid media, thus presenting no statistical difference between them.

In an analysis of the effect of liquid and solid consistencies of a culture medium on shoots of the yam cultivar Toyama Senju (*Dioscorea japonica* Thunb.), Kadota & Niimi (2004) obtained averages for shoot number of 1.7 and 2.5 and total fresh weight of 283.4 and 51.9 mg, respectively, and concluded that liquid medium is the best for micropropagation of shoots.

Table 5. Average values of number of senescent leaves (NSL), number of shoots (NS), number of mini-cuttings (NMC) and dry mass of the aerial part (DMAP; mg) of 'Inhame da Costa' (*Dioscorea rotundata* Poir) plants grown *in vitro* in solid and liquid media.

Consistency	NSL	NS	NMC	DMAP
Solid	0.16 b	1.47 a	4.85 a	29.33 b
Liquid	0.99 a	1.49 a	3.49 b	62.00 a

Averages followed by the same letter in the column do not differ by Tukey test at 5% probability.

This result was probably due to the increased availability of nutrients and water provided by this medium (Chen & Ziv, 2001), which, compared to solid media, has no physical resistance for nutrient dispersion. In addition, the greater contact of the roots and explants with the liquid medium favors the rate of nutrient assimilation by the cultured plant material (Ziv, 1995).

With the adjustment of the ideal culture medium for the species, the micropropagation rates can be increased, generating a larger number of plants in each sub-cultivation, which will contribute to a large-scale propagation, generating healthy plants suitable for planting in the field.

Conclusions

For the conditions of the experiment, the explants were 100% responsive in all treatments employed.

The 2GGC medium, in the liquid consistency, promoted greater plant development, being the most indicated for *in vitro* multiplication of 'Inhame da Costa' (*Dioscorea rotundata* Poir.).

Compliance with Ethical Standards

Author contributions: Conceptualization: LFSS, ASS, KCFS; Data curation: LFSS, JSSR; Formal analysis: CASL, MJSC, LFSS; Funding acquisition: ASS, CASL; Investigation: LFSS, JSSR, ASS, KCFS; Methodology: LFSS, JSSR, MJSC, KCFS; Project administration: ASS; Resources: KCFS, ASS; Supervision: KCFS, ASS, CASL; Validation: MJSC, ASS, KCFS; Visualization: LFSS, JSSR, KCFS; Writing – original draft: KCFS, ASS, JSSR; Writing – review & editing: KCFS, ASS.

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