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Influence of supplementation of the culture medium on *in vitro* development of *Jatropha curcas*

Influência da suplementação do meio de cultura no desenvolvimento *in vitro* de plântulas de *Jatropha curcas*

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Abstract

The Jatropha (*Jatropha curcas* L.) crop stands out for its socioeconomic potential, being an excellent alternative for biodiesel production. Therefore, this study aimed to standardize and optimize *in vitro* culture of Jatropha embryos and seeds by means of modifications with the addition of vitamin supplements and gibberellic acid in MS medium. We sought to obtain aseptic explants to be used in tissue culture and genetic transformation for the production of standardized seedlings. Two experiments were carried out on MS medium with sucrose and agar. In the first experiment, vitamin complex was added to the culture medium with embryos, at doses of 0.0; 0.5; 1.0; 2.0; 3.0 and 4.0 mL L^{-1}. The second experiment was carried out with seeds added with various doses of gibberellic acid (0.0; 1.0; 2.0; 4.0; 6.0 and 8.0 mg L^{-1}). The experiments were conducted in a completely randomized design with four replicates. Ten variables were analyzed in a two-week period. The results indicated that the addition of vitamins and gibberellic acid to the culture medium is not essential for germination and initial growth, but they provide important increases in seedling shoot and root development.

Additional keywords: gibberellic acid; Jatropha; micropropagation; vitamin complex.

Resumo

A cultura do pinhão-manso (*Jatropha curcas* L.) destaca-se pelo potencial socioeconômico, sendo uma excelente alternativa para a produção de biodiesel. Sendo assim, o objetivo deste trabalho foi a padronização e otimização de cultivo *in vitro* para embriões e sementes de pinhão-manso por meio de modificações com adição dos suplementos vitamínico e ácido giberélico em meio MS, visando a obtenção de explantes assépticos que serão utilizados na cultura de tecidos e transformação genética para a produção de mudas padronizadas. Foram realizados dois experimentos em meio MS com sacarose e ágar. No primeiro experimento, foram utilizados embriões com dosagens do complexo vitamínico (0,0; 0,5; 1,0; 2,0; 3,0 e 4,0 mL L^{-1}) acrescidas ao meio de cultura. O segundo experimento foi realizado com sementes adicionadas de várias dosagens de ácido giberélico (0,0; 1,0; 2,0; 4,0; 6,0 e 8,0 mg L^{-1}). Os experimentos foram conduzidos em delineamento inteiramente casualizado com quatro repetições, e após duas semanas, dez variáveis foram analisadas. Os resultados indicaram que a adição de vitaminas e de ácido giberélico ao meio de cultura não é essencial para a germinação e crescimento inicial, mas fornecem incrementos importantes no desenvolvimento da parte aérea e sistema radicular das plântulas.

Palavras-chave adicionais: ácido giberélico; complexo vitamínico; micropropagação; pinhão-manso.

Introduction

Jatropha curcas L. is an oleaginous species belonging to the family Euphorbiaceae and presents great potential for biodiesel production. It is a species of wide geographical distribution due to its rusticity and resistance to long droughts, pests and diseases, being found spontaneously in almost all intertropical regions (Ly et al., 2014).

This species is highlighted as a source of biofuel production by its high seed oil content, between 22% and 42% (Sunil et al., 2008). Regarding yield, Jatropha presents potential yields of 1200 to 1500 kg ha^{-1} of oil from the fourth year of cultivation (Laviola et al., 2014). Commercial Jatropha production to meet biodiesel production is already a reality in several countries, such as: Senegal, Zambia, Tanzania, Namibia, the Philippines and especially India (Attaya et al., 2012).

In view of the future commercial production of Jatropha in Brazil, large-scale propagation of elite materials is essential. The most suitable method for

large-scale seedling production is *in vitro* propagation, which is one of the most practical high-impact applications of tissue culture. However, especially for species of the genus *Jatropha*, this technique presents great challenges to be overcome, such as: determination of the explants most suitable for organogenesis, induction of development and/or elongation of shoots, induction of rooting, among others (Franco et al., 2014).

Although the principles of tissue culture are well defined, such as the basic composition of the culture media and the function of each growth regulator, each species requires targeted studies so that optimal conditions for its cultivation are known (Lopes et al., 2012). Studies evaluating the supplementation of the culture medium with carbohydrate sources and other additives that promote *in vitro* germination of Jatropha are essential not only for increasing the germination rate, but also for obtaining seedlings with great physiological potential (Nunes et al., 2008). The composition of the culture medium should encompass all minerals essential to plant nutrition, providing a carbon source in view of the reduced photosynthetic capacity of explants. Some growth-regulating vitamins, such as auxins and cytokinins, may also be incorporated into the medium to induce explant development (Carvalho et al., 2011).

Therefore, this study tested different amounts of supplements in *in vitro* culture, both with Jatropha embryos and seeds.

Material and methods

The experiments were carried out at the Laboratory of Biotechnology Applied to Plant Breeding, and the seeds used were collected from the *ex-situ* Germplasm Bank, both located in the Plant Production Department of the Faculty of Agrarian and Veterinary Sciences, Jaboticabal Campus.

During washing and disinfection in a beaker, the seeds were washed with water and neutral detergent three times, placed in a laminar flow chamber for five minutes in 70% ethanol and for twenty minutes in 2% sodium hypochlorite. The seeds were then sequentially rinsed three times in sterile water. After this process, the seed coat was removed, and asepsis was performed again.

MS culture medium (Murashige & Skoog, 1962) with sucrose (30 g L^{-1}) and agar (6 g L^{-1}) was used. The pH was adjusted to 5.8 and the culture medium was autoclaved at 120 °C and 1 atmosphere for 15 minutes. After cooling the medium to approximately 45 °C, doses of the previously sterilized supplements were added using a membrane filter with 0.22 µM porosity.

In experiment 1, six treatments corresponding to vitamin supplementation concentrations (0; 0.5; 1.0; 2.0; 3.0 and 4.0 mL L^{-1}) were evaluated in the medium. The experimental design was completely randomized, with four replicates, each replicate with 10 embryos. To obtain them, the seeds that had undergone asepsis and without integument remained in the water of the last rinse for about 20 hours to facilitate the withdrawal, which was performed in a laminar flow chamber with the aid of sterilized tweezers and scalpel.

As a supplement, was used a vitamin complex consisting of 0.008 mM nicotinic acid (vitamin B3), 0.55 mM myo-inositol, 0.03 mM thiamine (vitamin B1), 0.0049 mM pyridoxine (vitamin B6), 58.4 mM sucrose and water. These same components were suggested for embryo cultivation of *Butia capitata* (sour coconut) (Ribeiro et al., 2011). The embryos were individually arranged in 100-mL glass vials containing 25 mL of culture medium, which were sealed with polyvinyl chloride (PVC) film and taken to a growth room.

In Experiment 2, six treatments were evaluated, corresponding to the following gibberellic acid concentrations: GA3 (0; 1; 2; 4; 6 and 8 mg L^{-1}). The experimental design was completely randomized, with four replicates, each replicate with 24 seeds.

In a previous test, it was verified that the presence of seed integument prevented its germination. For this reason, the seeds that underwent aseptic treatment had the integument removed with the aid of sterilized pliers, and asepsis was performed again.

Subsequently, GA3 concentrations were added to the culture medium, according to each treatment. Three seeds were deposited in each of the 350-mL plastic vials containing 75 mL of medium, which were then sealed with a plastic cap and taken to the growth room.

The growth room where the *in vitro* cultures remained (both experiments) presents the following conditions: luminous intensity of 30 µmol m^{-2} s^{-1}, photoperiod of 12 hours and average temperature of 25°C.

The evaluations of experiments 1 and 2 were performed fifteen days after the establishment. The following were evaluated: percentage of germinated seeds (%G); seedlings with size 0 (%S0): those that did not grow; seedlings with size 1 (%S1): up to 1 cm; seedlings with size 2 (%S2): from 1 to 3 cm; seedlings with size 3 (%S3): with more than 3 cm; number of roots (NR); percentage of seedlings with roots greater than 1 cm (%SR>1); percentage of seedlings with roots smaller than 1 cm (%SR<1); and percentage of seedlings with leaves (%L).

Data were submitted to analysis of variance and the means were compared by the Tukey test at 5% probability. All statistical analyses were performed using the statistical program AgroEstat - Statistical Analysis System for Agronomic Tests (Barbosa & Maldonado, 2010).

Results and discussion

Experiment 1

The presence of vitamin complex was detrimental to the germination of embryos, since 100% of the embryos germinated in its absence. On the other hand, the highest concentration of the vitamin complex in MS medium gave the highest percentages of seedlings with leaves (Table 1, Figure 1a).

Table 1 - Percentage of germinated embryos (%G); percentage of seedlings with size 0 (%S0); with size 1 (%S1). with size 2 (%S2); and with size 3 (%S3); percentage of seedlings with root (%SR); total number of roots (%NR); percentage of seedlings with roots greater than 1 cm (% SR>1); with root less than 1 cm (%SR<1) and percentage of seedlings with leaves (%L) grown in MS medium with different concentrations of vitamin complex.

Concentrations (mL L^{-1})	%G	%S0	%S1	%S2	%S3	%SR	NR	%SR>1	%SR<1	%L
0.0	100.00a[1]	38.61a	18.33b	33.05ab	10.00bc	61.39b	19.50b	72.96a	27.03a	7.50ab
0.5	75.00cd	0.00b	13.84b	56.69a	29.46ab	100.00a	28.00ab	61.73a	38.26a	0.00b
1.0	80.00bc	0.00b	5.90b	46.82ab	47.27a	100.00a	35.00a	65.75a	34.25a	6.25ab
2.0	77.50bc	0.00b	15.62b	48.66ab	35.71a	100.00a	30.25a	70.98a	30.68a	19.19ab
3.0	65.00d	0.00b	27.38b	34.52ab	38.09a	100.00a	26.00ab	75.14a	24.86a	15.47ab
4.0	87.50b	0.00b	68.75ab	25.69b	5.55c	94.44a	30.00a	67.57a	32.42a	25.69a

(1) Means followed by equal letters in the column do not differ by Tukey test ($p > 0.05$).

The absence of vitamin complex negatively influenced seedling shoot length (Figure 1c), since there was a higher percentage of plants in the %S0 class, while treatments that had vitamins added showed a higher percentage of seedlings with heights classified as %S2 and %S3 (Table 1). Notwithstanding, with the highest concentration of the vitamin complex, most seedlings had a size of up to 1 cm, showing that excess vitamins can also affect seedling development. According to Rodrigues et al. (2006), excess sucrose in the culture medium can be harmful since it inhibits chlorophyll synthesis, reducing the photosynthetic capacity of tissues. Isolating the %S3 parameter, which comprises the seedlings that presented the best shoot length, concentrations of 0.5 to 3.0 mL L^{-1} showed no significant difference. In contrast, the treatments without vitamin supplement and with the highest concentration presented the smallest percentage of seedlings larger than 3 cm. A similar result was found by Lopes et al. (2012), when evaluating the development of Jatropha embryos as a function of sucrose concentrations in the culture medium. The authors observed that the treatments with intermediate concentrations presented more elongated seedlings, while extreme treatments affected plant growth. On the other hand, the result differs from that obtained by Nunes et al. (2008), where increased sucrose concentrations in the culture medium provided linear growth in the shoot length of Jatropha seedlings.

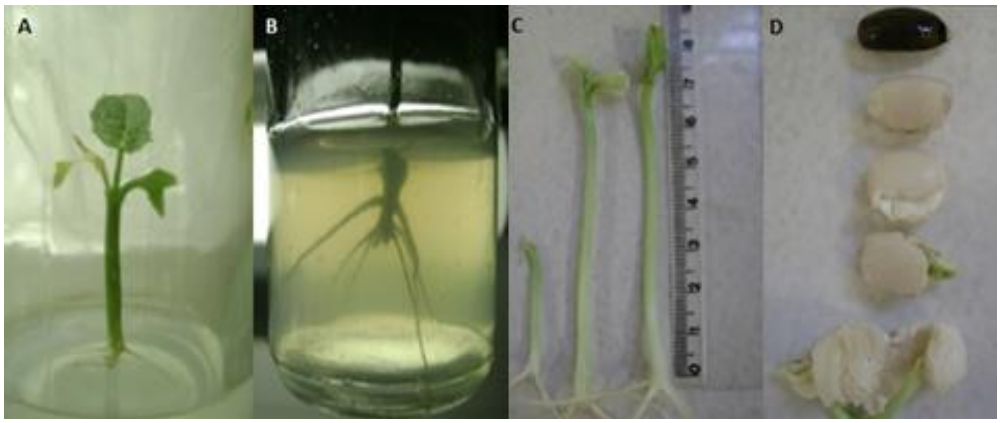

Figure 1 - Embryos and seeds of Jatropha in MS medium supplemented with vitamins and GA3 respectively. A: seedling developed from embryos with leaf presence; B: details of the root system of seedlings; C: seedlings with different lengths of the aerial part; D: seeds with tegument, without the integument and the others germinated.

Regarding rooting, analyzed by the variables %SR and NR, the presence of vitamin complex was favorable when compared to the non-addition of vitamins (Table 1).

The addition of vitamins favored the appearance of roots, highlighting the concentration of 1.0 mL L^{-1}, which allowed a significant increase in the number of roots when compared to the treatment without addition of vitamins. The high number of roots in the seedlings can be explained by the presence of vitamins, which induced the rapid growth of shoots, providing intense production of auxin. This, in turn, was translocated to the plant base, stimulating rhizogenesis, a common fact in the processes that involve tissue culture (Kochhar et al., 2008; Barbosa et al., 2011).

The addition of supplements was not efficient for root elongation (Figure 1b). In all treatments, the roots of most seedlings reached a length higher than 1 cm, even in the absence of vitamin complex,

evidencing that elongation was not influenced by the presence of vitamins. Different sucrose concentrations also did not influence root elongation of Jatropha seedlings in the study by Lopes et al. (2012).

Experiment 2

It was observed that seed germination (Figure 1d) was not dependent on the GA3 concentrations used (Table 2). The fact that the seeds germinated at all concentrations of gibberellic acid may be related to the presence of the hormone in the seeds, with the existing concentration being already sufficient to metabolically support germination. Unlike these results, Peixoto et al. (2011) observed an increase in the germination percentage of castor bean seeds subjected to gibberellin treatments.

Table 2 - Percentage of germinated seeds (%G); percentage of seedlings with size 0 (%S0); with size 1 (%S1); with size 2 (%S2); with size 3 (%S3); percentage of seedlings with root (% SR); total number of roots (NR); percentage of seedlings with roots greater than 1 cm (%SR> 1); less than 1 cm (%PR<1) and percentage of seedlings with leaves (%L) grown in MS medium with different concentrations of gibberellic acid.

Concentrations (mL L^{-1})	%G	%S0	%S1	%S2	%S3	%PR	NR	%SR>1	%SR<1	%L
0.0	89.58a[1]	33.23ab	35.47a	16.85a	14.43b	40.91b	26.00a	63.84ab	36.16bc	0.00a
1.0	88.54a	25.06ab	3.85b	24.39a	46.69a	70.05a	33.50a	74.23a	25.77c	1.47a
2.0	89.18a	19.16b	3.91b	21.85a	55.06a	63.41ab	34.75a	35.90c	64.09a	3.84a
4.0	86.80a	24.26ab	13.99b	17.24a	44.49ab	68.23a	38.00a	54.53abc	45.47abc	1.13a
6.0	98.81a	36.52ab	0.00b	12.50a	50.97a	59.44ab	33.54a	49.97bc	53.84ab	2.91a
8.0	90.87a	44.10a	6.11b	13.05a	33.95ab	50.06ab	23.75a	32.10c	67.89a	0.00a

(1) Means followed by equal letters in the column do not differ by Tukey test ($p > 0.05$).

The presence of GA3 in the medium positively influenced seedling shoot growth. According to Table 2, for the variable %S3, the presence of GA3 was efficient, despite the small variation between the treatments with concentrations from 0 to 4 mg L^{-1}. The best result was obtained with 2.0 mg L^{-1}, where 55% of the seedlings reached sizes greater than 3 cm. However, at the highest dose (8.0 mg L^{-1}), it was verified that more than 40% of the seedlings did not grow.

Regarding the rooting of seedlings, according to %SR and NR, the results point to a progressive increase in the number of roots between 1.0 mg L^{-1} and 4.0 mg L^{-1}, with a decrease in NR at higher concentrations. The percentage of seedlings with roots (%SR) also showed significant difference between the application or non-application of GA3 (Table 2).

It is assumed, then, that a high GA3 concentration is detrimental to root development. Several authors have demonstrated that the effects of GA3 can be contradictory, often inhibiting (Carvalho et al., 2009) and other times stimulating (Soares et al., 2012) root organogenesis.

Regarding root length, for the variable %SR>1, the absence of GA3 provided a better result when compared to the concentrations tested, evidencing that increased GA3 concentration impairs root elongation. This can also be noticed in the results for the variable %SR<1, where the highest concentration yielded the highest number of seedlings with roots smaller than 1 cm.

Thus, there was a better effect of GA3 on rooting (%SR and NR) than on root elongation. This fact may be related to the conditions of the culture medium, allowing a high incidence of light. Nunes et al. (2008) found that the addition of activated carbon at certain concentrations may be beneficial to root development by simulating the dark condition, in which roots normally develop better. According to George et al. (2008), rooting success depends not only on root length but also on the number and quality of roots formed.

For the variable %L, there was no significant difference between treatments (Table 2).

Conclusion

The addition of 1.0 mL L^{-1} of vitamins to MS medium favors rhizogenesis and shoot elongation of Jatropha embryos cultured *in vitro*.

At the concentrations of 2.0 and 4.0 mg L^{-1}, gibberellic acid favors shoot development and root formation, respectively, in Jatropha seeds cultured *in vitro*.

Supplementation of the MS medium with vitamin complex above 4.0 mL L^{-1} or GA3 above 8.0 mg L^{-1} is detrimental to Jatropha seedlings.

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