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Activated charcoal in the control of oxidation and *in vitro* growth of *Dioscorea* spp. nodal segments

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ABSTRACT. *In vitro* oxidation is a problem for some herbaceous and woody species and can cause darkening of tissues and consequently death of explants and plants Therefore, this study aimed to assess the effect of activated charcoal on in vitro yam cultivation, aiming at reducing or eliminating explant oxidation and optimizing the growth of the genotypes *Dioscorea alata* var. *purpurea* (Roxb.) A. Pouchet and *Dioscorea rotundata* Poir. Nodal segments of approximately 1 cm, extracted from plants previously grown in vitro, were introduced into test tubes containing 10 mL of 2GGC culture medium, plus 30 g L^{-1} sucrose, solidified with 2.2 g L^{-1} Phytagel® and pH adjusted to 5.8 before autoclaving, containing activated charcoal doses of 0, 1, 2, 3 and 4 g L^{-1}. Plants were maintained for 90 days in a growth room, with temperature of 27 ± 1ºC, photon flux density of 30 µmol m^{-2} s^{-1} and photoperiod of 16 hours, after which their development variables were evaluated. Activated charcoal, at the concentration of 4 g L^{-1} considerably promoted the best development of plants, and the species *D. alata* var. *purpurea* showed higher means for all variables studied.

Keywords: phenolic compounds; antioxidants; tissue culture; in vitro multiplication.

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Introduction

Yam (*Dioscorea* spp.) belongs to the family Dioscoreaceae and the genus *Dioscorea*, encompassing more than 600 species, all monocotyledons (Fotso, Sandrine, Désiré, François, & Denis, 2013). Of these species, the most important ones are *Dioscorea rotundata* Poir., *Dioscorea alata* L., *Dioscorea bulbifera* L., *Dioscorea opposita* Thunb., *Dioscorea esculenta* (Lour.) Burkill and *Dioscorea dumetorum* (Kunth) Pax (Jyothy et al., 2017).

The species *D. alata* and *D. rotundata* are considered the most economically important, because they are the most consumed and are widely distributed worldwide (Arnau, Némorin, Maledon, & Abraham, 2009; Salcedo-Mendoza, García-Mogollón, & Salcedo-Hernández, 2018). The species *D. alata* is native to Asia and produces tubers of light brown to dark brown color and white to cream mass, while *D. rotundata*, native to Africa, produces tubers of brown color and white mass; both belong to the same botanical section, Enantiophyllum (Arnau et al., 2009; Vega, 2012).

Yam provides food security and income for population of Africa, the Caribbean, Asia and America, and is therefore considered of great economic importance (Baah, Maziya-Dixon, Asiedu, Oduro, & Ellis, 2009). With an estimated production of about 50 million tons per year, yam occupies the fourth position in the world in importance among root- and tuber-producing plants (García et al., 2011). Brazil is the second largest producer of yam in Latin America (Simões, Lino, da Silva Souza, de Oliveira, & da Silva Ledo, 2016). Yam tubers are rich in carbohydrates, vitamins and minerals (Baah et al., 2009) and, for having medicinal properties, are used in pharmacology, in the preparation of stimulants, tonics, carminatives and expectorants (Behera, Sahoo, & Prusti, 2009).

Despite the economic and nutritional importance, yam is a crop that has undergone few improvements in relation to its production and management, with predominance of the conventional vegetative propagation technique for its reproduction, which increases the rate of contamination by diseases and resulting in depreciation of the tuber (Ahanhanzo, Agbangla, Gandonou Ch, Dansi, & Dramane, 2008; Vega, 2012). Thus,

in each production cycle, seed tuber that may be contaminated by diseases and pests are used, due to lack of quality planting material and the high cost for acquiring healthy seeds.

In vitro cultivation of yam can be a way to obtain planting material with phytosanitary quality, overcoming the problems encountered in conventional propagation, besides favoring its multiplication quickly and adequately (Ahanhanzo et al., 2008; Behera et al., 2009; Das, Choudhury, & Mazumder, 2013; Agbidinoukoun, Ahanhanzo, Adoukonou Sagbadja, Adjassa, & Agbangla, 2013). *In vitro* clonal propagation can produce pathogen-free seeds with physiological and sanitary quality, providing a uniform plant material, with more vigor, higher propagation speed and, consequently, a greater number of regenerated plants, which would considerably increase yield (Quintero, Polo, Jarma, & Espitia, 2003; Cabrera Jova, 2009).

However, in *in vitro* cultivation, it is necessary to adapt protocols according to each species, because several factors, such as culture medium, growth regulators (Quintero et al., 2003; Ezeibekwe, Ezenwaka, Mbagwu, & Unamba, 2009; Simões et al., 2016), antioxidants (Melo, Cabral, Resende, & Andrande, 1998; Borges et al., 1999; García & Tabarez, 2008), carbon source (Ovono, Kevers, & Dommes, 2007; Souza, Bertoni, França, & Pereira, 2011), light, temperature, type and source of explants (Behera et al., 2009), will influence the *in vitro* establishment (García, Abeal, Rodríguez, & Rodríguez, 2009), growth, multiplication (Souza et al., 2011) and conservation of plants (Borges García, Alarcón Sánchez, Malaurie, Hernandez Jerez, & Silva Pupo, 2009).

Among these factors, for some species of the genus *Dioscorea*, one of the most frequent and limiting *in vitro* growth, and cause the death of the explant is the oxidation of phenolic compounds, because when the tissue is sectioned a metabolic reaction occurs (Azofeifa-Delgado, 2009) and the affected cells exude substances that cause darkening of the explant and culture medium (Melo et al., 1998). To prevent or reduce the effects of *in vitro* oxidation, some antioxidant substances are used, such as activated charcoal, cysteine, polyvinylpyrrolidone (PVP), ascorbic acid and citric acid, which can be polyphenol oxidase inhibitors or act as adsorbents (Melo et al., 1998; Paiva, Paiva, & Pasqual, 2007; García & Tabarez, 2008; Azofeifa-Delgado, 2009).

In this respect, activated charcoal can exert beneficial and harmful effects on the *in vitro* cultivation of tissues because, according to García and Tabarez (2008), its addition to the culture medium can interfere in the absorption of nutrients considered essential, such as nitrogen, and in the action of endogenous hormones, which are fundamental for bud formation. However, due to the adsorption of hormones exuded by plants and toxic metabolites, activated charcoal acts as a potential antioxidant, besides controlling the release of metabolites by promoting a dark environment for explants inoculated in the culture medium (Fagundes, Moreira, Ramm, Schuch, & Tomaz, 2017).

Thus, this study aims to assess the effect of activated charcoal on *in vitro* cultivation of yam, aiming at reducing oxidation and optimizing the growth of the genotypes *D. alata* var. *purpurea* and *D. rotundata*.

Material and methods

The experiment was conducted at the Tissue Culture Laboratory of Embrapa Cassava and Tropical Fruits, in Cruz das Almas, Bahia, Brazil. For this, nodal segments of approximately 1 cm were extracted from plants of *D. rotundata* Poir. and *D. alata* var. *purpuranda* (Roxb.) A. Pouchet, previously grown *in vitro* and introduced into test tubes (2.5 cm x 15 cm) containing 10 mL of 2GGC medium (Doukoure, Ahoussou, Zoundjihekpon, & Tio-Toure, 2000). In this medium, activated charcoal was added at doses of 0, 1, 2, 3 and 4 g L^{-1}, plus 30 g L^{-1} sucrose, solidified with 2.2 g L^{-1} phytagel® and with pH adjusted to 5.8 before autoclaving.

The experiment was installed in a completely randomized design, in a 5 x 2 factorial scheme (five concentrations x two genotypes), containing 12 replicates per treatment. Each experimental plot consisted of a test tube containing a nodal segment.

After introduction into the culture medium, the explants were kept in the growth room, with temperature conditions of 27 ± 1ºC, photon flux density of 30 µmol m^{-2} s^{-1} and photoperiod of 16 hours.

After 90 days of *in vitro* cultivation, the following variables were evaluated: shoot height (SH), in cm, number of green leaves (NGL), number of shoots (NS), number of nodal segments (NNS), number of roots (NR), shoot fresh mass (SFM), root fresh mass (RFM), shoot dry mass (SDM), root dry mass (RDM), in mg, and length of the longest root (RL), in cm. For dry mass determination, the plant material was placed in a forced air circulation oven at a temperature of 60ºC, until it reached constant weight.

The obtained data were subjected to the F test of the analysis of variance, with the statistical program R, version 3.4 (R Core Team, 2016), using the ExpDes.pt package (Ferreira, Cavalcanti, & Nogueira, 2018). For the means of antioxidant concentrations, polynomial regression models were fitted, and the genotype means

were compared by F test at 5% probability level. For the variables that did not meet the assumptions of the analysis of variance, the data were transformed to $\sqrt{x + 0.5}$.

Results and discussion

Under the experimental conditions studied, the nodal segments in general showed 95.83% of regeneration. However, Simões et al. (2016) obtained 100% of surviving plants in MS medium composed of minerals and vitamins, plus 100 mg L^{-1} inositol, 20 mg L^{-1} cysteine, 0.20 mg L^{-1} naphthaleneacetic acid (NAA), 0.08 mg L^{-1} gibberellic acid (GA_3), supplemented or not with 0.15 mg L^{-1} of 6-benzylaminopurine (BAP), working with the species *D. rotundata*. While Verde et al. (2021) obtained an average of 97.5% of explant regeneration of *D. alata var. purpurea* and *D. rotundata*, when subjected to different concentrations of the antioxidants ascorbic acid and polyvinylpyrrolidone.

The analysis of variance by F test (Table 1) showed that, for shoot height (SH), number of green leaves (NGL), number of nodal segments (NNS), number of roots (NR), and shoot and root fresh and dry masses (SFM, SDM, RFM and RDM), there were significant effects of the individual factors. For the length of the longest root (RL), there was a significant effect only for the genotype factor, while for the number of shoots (NS) the individual factors were not significant. In the interaction between factors (activated charcoal concentration x genotype), there was a highly significant effect only on shoot height (SH).

The coefficients of variation ranged between 12.53%, for shoot height (SH), and 40.06%, for root dry mass (RDM) (Table 1). Simões, Lino, Silva Souza, Oliveira, and Silva Ledo (2014), found CVs between 29.47 and 44.13% in the multiplication of *D. rotundata*, under the same conditions of control of temperature, light and photoperiod as the present study, but in MS medium.

Table 1. Summary of the analysis of variance for the growth variables of the genotypes *Dioscorea alata var. purpurea* and *Dioscorea rotundata*, subjected to different doses of activated charcoal in 2GGC medium.

SV	DF	SH	NGL	NS	NNS	NR	SFM	SDM	RFM	RDM	RL
AC	4	3.22^{**}	5.16^{**}	0.11^{ns}	4.88^{**}	1.58^{**}	531.37^{**}	39.43^{**}	0.46^{**}	0.26^{**}	0.65^{ns}
GNT	1	3.94^{**}	11.04^{**}	0.00^{ns}	9.73^{**}	7.73^{**}	910.05^{**}	24.95^{**}	7.92^{**}	2.72^{**}	67.90^{**}
AC*GNT	4	0.61^{**}	0.33^{ns}	0.05^{ns}	0.44^{ns}	0.82^{ns}	51.42^{ns}	4.83^{ns}	0.03^{ns}	0.01^{ns}	0.29^{ns}
Error	105	0.12	0.38	0.06	0.25	0.35	30.64	1.98	0.10	0.05	0.66
Mean		7.43	10.61	1.90	6.14	4.03	509.89	39.77	41.39	3.91	11.21
CV (%)		12.53	19.02	16.24	20.15	29.57	25.97	23.21	22.99	40.06	25.11

SV = source of variation; DF = degrees of freedom; AC = activated charcoal; GNT = genotype; SH = shoot height (cm); NGL = number of green leaves; NS = number of shoots; NNS = number of nodal segments; NR = number of roots; SFM = shoot fresh mass (mg); SDM = shoot dry mass (mg); RFM = root fresh mass (mg); RDM = root dry mass (mg); RL = length of the longest root (cm). **; *significant by F test at 1 and 5% probability levels, respectively.

The polynomial regression models, the optimal concentrations of activated charcoal and the estimated values of each variable are presented in Table 2. The coefficients of determination (R^2) obtained ranged from 58.93% for number of roots to 96.95% for shoot height, in the interaction between activated charcoal doses and the genotype *D. alata* var. *purpurea*. In the micropropagation of *D. rotundata*, Simões et al. (2016) studied the effect of BAP concentrations on M1 medium (vitamins of MS) and M2 (vitamins of MS, plus 100 mg L^{-1} inositol, 20 mg L^{-1} cysteine, 0.20 mg L^{-1} NAA and 0.08 mg L^{-1} GA_3 and obtained for shoot height coefficients of determination of 73.44 and 79.98% for M1 and M2 media, respectively. The coefficient of determination of the equations is an important factor, as it will reflect how much the linear or polynomial model adjusts to the observed values. Thus, the closer to 100%, the greater is the reliability of the model being represented.

According to the values obtained from the linear equations, the optimal dose of activated charcoal was 4 g L^{-1} for the variables shoot height, number of green leaves, number of nodal segments, number of roots, and shoot and root fresh and dry masses of the genotype *D. rotundata* (Table 2). Poornima and Ravisharnkar (2007) used a similar concentration, 3 g L^{-1} of activated charcoal, in the *in vitro* propagation of *D. oppositifolia* (Linn) and *D. pentahylla* (Linn). Contrasting these results, Sêdami et al. (2017) used Galzy glutamine (2 GG) culture medium, without and with supplementation with activated charcoal (3 g L^{-1}), in the *in vitro* cultivation of *D. cayenensis-rotundata,* and obtained the best results for the variables number of leaves, number of roots, stem height and average length of the main root, in the absence of activated charcoal. However, according to these authors, the addition of activated charcoal in the culture medium contributed to a greater viability of plant tissue, highlighting that the presence of this antioxidant left plants with greener leaves, unlike the medium without charcoal, which had achlorophyllous leaves with the progression of the *in vitro* conservation of that genotype.

Table 2. Regression equations, coefficients of determination (R^2), optimal doses (OD) of activated charcoal (g L^{-1}) and estimated values (EV) of development variables of the genotypes *Dioscorea alata var. purpurea* and *Dioscorea rotundata*.

	Genotypes	Equation	R^2 (%)	OD	EV
Shoot height (cm)					
Activated charcoal (genotype)	*D. alata* var. *purpurea*	$\hat{y}^{**} = -0.544x^2 + 3.1964x + 5.3374$	96.95	2.93	10.03
	D. rotundata	$\hat{y}^{**} = 1.3188x + 3.7664$	92.87	4	9.04
Number of green leaves					
Activated charcoal		$\hat{y}^{**} = 1.8491x + 6.8602$	86.24	4	14.26
Number of nodal segments					
Activated charcoal		$\hat{y}^{**} = 1.3548x + 3.4006$	89.38	4	8.82
Number of roots					
Activated charcoal		$\hat{y}^{**} = 0.5002x + 3.008$	58.93	4	5.01
Shoot fresh mass (mg)					
Activated charcoal		$\hat{y}^{**} = 121.59x + 264$	90.67	4	750.36
Shoot dry mass (mg)					
Activated charcoal		$\hat{y}^{**} = 9.3209x + 20.895$	92.01	4	58.18
Root fresh mass (mg)					
Activated charcoal		$\hat{y}^{**} = 5.0615x + 30.651$	66.64	4	50.90

**significant at 1% probability level by the F test of ANOVA.

The addition of activated charcoal is fundamental for a better development of yam plants, as evidenced by García et al. (2011), who added 0.5 g L^{-1} of the antioxidant and obtained a considerable increase in the development of plants *in vitro*, which affected all variables studied. According to Corrêa, Pinto, Bertolucci, Reis, and Souza (2003), despite not being a growth regulator, activated charcoal modifies the composition of the culture medium, promoting, in some circumstances, improvement of plant growth *in vitro*.

The regeneration of nodal segments using activated charcoal contributes to a better tissue development, which may result in a higher percentage of shoot recovery in the treatments with charcoal addition (Borges et al., 2004). This positive effect on the growth of yam nodal segments was also confirmed by Polzin et al. (2014), in both solid and liquid media. Thus, as observed in this study, the presence of activated charcoal promoted higher means in the variables studied, compared to the culture medium without its addition.

There was a quadratic relationship for the shoot height of the genotype *D. alata* var. *purpurea* and an increasing linear relationship for the genotype *D. rotundata*. According to Table 2, it can be observed that the increase in the doses of activated charcoal promoted greater plant growth, with optimal dose of 4 g L-¹ and an estimated value of 9.04 cm for *D. rotundata*. For *D. alata* var. *purpurea*, the maximum estimated height was 10.03 cm, obtained with the estimated optimal dose of 2.93 g L^{-1} of activated charcoal (Table 2). Agbidinoukoun et al. (2013) observed that the increase in the concentration of activated charcoal to 3 g L^{-1} reduced the average height of three yam accessions. However, among the different concentrations studied, these authors did not obtain significant differences for this variable. Polzin et al. (2014) obtained a positive effect on plant length using MS medium with the addition of 3 g L^{-1} of activated charcoal.

Some studies report the use of activated charcoal in the *in vitro* conservation of *Dioscorea* species at concentrations of 2 g L^{-1} (Borges et al., 2004; Borges García et al., 2009; Abeal et al., 2009) and 3 g L^{-1} (Sêdami et al., 2017). This indication is due to the fact that, apparently, charcoal retains nutrients that are slowly absorbed by plants, allowing their continued presence in the medium; in addition, the use of charcoal produces plants with a higher number of nodes and lower height (Sêdami et al., 2017). However, in this study, the use of activated charcoal did not promote significant results for the number of shoots and increased the average plant height.

The number of green leaves showed a linear behavior, with an optimal dose of 4 g L^{-1} of activated charcoal and an estimated value of 14.26 green leaves (Table 2). Studying yam accessions of the *D. cayenensis - D. rotundata* complex and *D. alata*, Agbidinoukoun et al. (2013) obtained the highest means of the number of leaves in the absence of activated charcoal, with reduction at the concentration of 3 g L^{-1}. Diverging from these results, García and Tabarez (2008) found no significant results for this variable, as well as for plant height at charcoal doses of 0.5, 1.0, 2.5 and 2.0 g L^{-1} in *D. alata* L., after 6 weeks of *in vitro* cultivation of *D. alata*.

The optimal dose for the number of nodal segments is 4 g L^{-1} of activated charcoal, with an estimated value of 8.82 nodal segments (Table 2). Simões et al. (2014) did not obtain better development of nodes/buds with the addition of activated charcoal in *D. rotundata*. In contrast to this result, in *D. alata*, García and Tabarez

(2008) observed a decrease in the number of nodal segments with the addition of 2 g L^{-1} of activated charcoal to the culture medium. The same authors state that the appropriate multiplication rate, per subculture, of the yam plant material should be equal to or greater than 2.5 nodal segments. Therefore, considering the value obtained in this study after 12 weeks, it can be affirmed that in 2GGC medium, with the use of the appropriate dose of activated charcoal (4 g L^{-1}), it is possible to achieve optimal levels for the rapid multiplication of the yam genotypes studied, given that the estimated value (8.82 segments) was 3.5 times higher than that indicated by García and Tabarez (2008).

For the number of roots, the optimal dose was also 4 g L^{-1} of activated charcoal, with an estimated value of 5.01 roots per plant (Table 2). Simões et al. (2014), in *D. rotundata*, obtained a higher number of roots with the addition of 1 g L^{-1}, compared to the absence of activated charcoal and dose of 0.5 g L^{-1}, in the culture medium with ½ MS. Agbidinoukoun et al. (2013) obtained, after 8 months of *in vitro* cultivation, a greater number of roots with 3 g L^{-1} of this antioxidant. According to these authors, the high level of rooting obtained is not influenced by the type of medium, but by the addition of activated charcoal. Studies carried out by Feyissa, Welander, and Negash (2005), Yakoub-Bougdal, Chérifi, and Bonaly (2007) and Kamal and Sayyed (2011) confirmed that the addition of charcoal has positive effects on rooting, not only in species of the genus *Dioscorea*, but also in other plant species. Activated charcoal is involved in a series of activities, both stimulant and inhibitory, including the release of substances present in its constitution, which, in addition to darkening, promote changes in the composition of the nutrient medium with the adsorption of vitamins, metal ions and plant regulators, including abscisic acid and ethylene, hence favoring the *in vitro* growth (Sharma, Trivedi, & Purohit, 2012).

The highest means of shoot fresh mass (750.36 mg) and shoot dry mass (58.18 mg) were obtained at the optimal dose of 4 g L^{-1} of activated charcoal (Table 2). Evaluating fresh mass after 8 months, Agbidinoukoun et al. (2013) found considerable increments with 3 g L^{-1} of activated charcoal in Galzy medium supplemented with glutamine, since the means of fresh mass were equal to 179.06 and 252.80 mg for the Dcr28 and Dcr164 accessions, which belong to the *D. cayanenses – D. rotundata* complex, respectively, and to 205.60 mg for the accession Da93G1, which belongs to the species *D. alata*. Thus, these values are well below the mean obtained in the present study after only 3 months, with the activated charcoal concentration of 4 g L^{-1}.

For root fresh mass, the optimal dose of activated charcoal was also 4 g L^{-1}, with an estimated value of 50.90 mg (Table 2). For root dry mass, a high R^2 value with biological significance was not obtained. Differing from these results, Corrêa et al. (2003) obtained higher root dry mass in the absence of activated charcoal in sweet potatoes (*Ipomoea batatas* L. Lam). However, these authors state that activated charcoal is used to improve plant rooting, since it has the ability to block light, with the darkening of the culture medium, favoring the development of the root system.

Although it is not possible to evaluate the degree of oxidation *in vitro* with the presence of activated charcoal, it can be stated that its addition to the culture medium, at concentrations above the usual dose of 1 g L^{-1}, promoted improvements in the growth variables evaluated. Melo et al. (1998) observed little oxidation or necrosis, with the addition of activated charcoal, in nodal segments of *D. cayenensis* Lam. There is a significant importance of the use of activated charcoal in the culture medium, as it is capable of reducing or eliminating the phenolization of explants (Abeal et al, 2009; Azofeifa-Delgado, 2009). Activated charcoal, as previously seen, acts not only on the adsorption of phenolic substances, but also on the establishment of a darkened environment, adsorption of undesirable/inhibitory substances, growth regulators and organic compounds, or, on the other hand, on the release of growth-promoting substances present in or adsorbed by it (Azofeifa-Delgado, 2009; Polzin et al., 2014).

In relation to plant growth, it can be observed that the species *D. alata* var. *purpurea* shows the highest means for all variables analyzed (Table 3). Agbidinoukoun et al. (2013) found effect of the genotype on root length, since the observed means were equal to 3.6 cm for an accession of *D. alata* and 1.5 cm for two accessions of the *D. cayenensis - D. rotundata* complex.

Regarding shoot height, the trend of the means was similar to that observed for the other variables, when activated charcoal promoted the elongation of plants of both yam genotypes (Table 4), a result similar to that found by Mittal, Devi, and Gosal (2016) in a study conducted with three varieties of sugarcane. The species *D. alata* var. *purpurea* showed higher means than *D. rotundata* at all concentrations of activated charcoal and even statistically differing at the activated charcoal doses of 1 and 2 g L^{-1}.

Table 3. Means of development variables for the genotypes *Dioscorea alata* var. *purpurea* and *Dioscorea rotundata*, subjected to different doses of activated charcoal in the 2GGC culture medium for 90 days.

Variables	Genotypes	
	D. alata var. *purpurea*	*D. rotundata*
Number of green leaves	12.86 a	8.40 b
Number of nodal segments	7.72 a	4.59 b
Number of roots	5.05 a	3.02 b
Shoot fresh mass (mg)	647.49 a	374.66 b
Shoot dry mass (mg)	46.23 a	33.42 b
Root fresh mass (mg)	61.21 a	18.28 b
Root dry mass (mg)	5.35 a	2.24 b
Length of the longest root (cm)	16.14 a	5.47 b

Means followed by different letters in the same row differ by F test ($p<0.05$).

Table 4. Means of shoot height (cm) for the genotypes *Dioscorea alata* var. *purpurea* and *Dioscorea rotundata* subjected to different doses of activated charcoal in 2GGC culture medium for 90 days.

Genotypes	Activated charcoal (g L^{-1})				
	0	1	2	3	4
	Shoot height (cm)				
D. alata var. *purpurea*	5.44 a	7.62 a	10.04 a	9.76 a	9.48 a
D. rotundata	4.33 a	4.12 b	5.74 b	9.71 a	8.12 a

Means followed by the same letter in each column do not differ statistically from each other by the F test ($p<0.05$).

Conclusion

Activated charcoal, at the concentration of 4 g L^{-1}, promoted better development of the variables studied in both genotypes, and the species *D. alata* var. *purpurea* had higher means than *D. rotundata*.

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