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Humic acids and brassinosteroid application effects on pineapple plantlet growth and nutrition during the aclimatization phase

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Humic acid and brassinosteroid applications may be an alternative to decrease the pineapple plantlet acclimatization in *in vitro* cultivation, since promising results have been observed when these substances were independently applied in other propagation methods. In this sense, the aim of the present study was to evaluate the effects of humic acids and brassinosteroid application on 'BRS Vitória' pineapple plantlets grown from *in vitro* cultivation during acclimatization. A randomized block design was used in a 5x2x4 factorial scheme, at five brassinosteroid doses (0, 0.25, 0.50, 0.75, 1.0 mg L^{-1}) in the presence and absence of humic acids during four sampling periods (60, 90, 120 and 150 days after transplanting), with five replicates for each treatment. BIOBRÁS-16 was used as the brassinosteroid source, and the organic soil conditioner Agrolmin HF® was used as the humic acid source. Plantlets were collected for evaluation every 30 days from 60 days after transplanting. The number of plantlet leaves, length and root mass were higher in the humic acid treatment without brassinosteroid application. Leaf, nitrogen, phosphorus, potassium, calcium and magnesium contents were of 13.04, 1.77, 40.2, 8.79 and 3.17 mg kg^{-1}, respectively. Nitrogen and potassium contents in the plantlets decreased, while phosphorus contents increased as a function of acclimatization time, regardless of treatment.

Key words: *Ananas comosus* var. comosus, propagation, *in vitro*.

INTRODUCTION

The most planted pineapple cultivars in Brazil are the Pérola and Smooth Cayenne cultivars (AGRIANUAL, 2015), both susceptible to fusariosis, a disease caused by the *Fusarium guttiforme* fungus and the main

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pineapple cultivar disease in the country, responsible for the loss of up to 40% of total pineapple production. In recent years, several research groups have been developing resistant cultivars. In this context, the 'BRS Vitória' pineapple is a promising alternative, since it displays several favorable agronomic traits (Ventura et al., 2007; Ventura et al., 2009).

Specialized biofactories already commercialize 'BRS Vitória' pineapple plantlets that originated from the tissue culture technique. This is an indispensable tool for the availability of matrices throughout the national territory, since it is possible to produce large amounts of excellent quality homogeneous plantlets in a short time and in small space (Berilli et al., 2011). However, it is worth mentioning that this type of plantlet is still not very accessible to Brazilian farmers, due to its high cost (Moraes et al., 2010).

The final cost of a propagule obtained by this technique can reach a cost of up to 10 times more than conventional plantlets. In this context, the development of new technologies that improve this technique is fundamental, in order to offer better standards and cheaper plantlets. The application of certain substances, such as humic acids and brassinosteroids, can serve this purpose, since some studies using these substances, applied in isolated form, in pineapples have obtained promising results (Catunda et al., 2008; Baldotto et al., 2009; Freitas et al., 2012; Santos et al., 2014).

The main brassinosteroid effects are cell stretching and expansion, resistance to stress and delay of leaf abscission (Fujioka and Saakurai, 1997). Deficiencies in the biosynthesis or perception of these hormones generates dwarf plants with a dark green color, presenting leaf epithelia with reduced or no fertility and developmental delays (Bishop and Koncz, 2002).

Humic acid applications can provide higher plant growth and productivity by positively influencing ion transport and facilitating nutrient uptake (Nardi et al., 2002). In addition, these compounds increase respiration and the rate of Krebs cycle reactions, resulting in higher ATP production (Canellas et al., 2002), while also supplying nutrients to plants through their mineralization (Cordeiro et al., 2010).

These technological approaches may lead to increased pineapple plantlet growth in nurseries and may impact the biofactory production system, therefore, making these plantlets more affordable to farmers. In this context, the aim of the present study was to evaluate the effect of humic acid and brassinosteroid application on 'BRS Vitória' pineapple plantlet growth and leaf nutrient content during the acclimatization period.

MATERIALS AND METHODS

The experiment was conducted under greenhouse conditions. During the experimental period, temperature and relative humidity data were collected by the WATCH DOG Weather Station (Weather Station, Spectrum Technologies, Inc), programmed to perform readings at one- hour intervals (Figure 1).

The experimental design comprised a randomized block design in a 5×2×4 factorial scheme, comprising five brassinosteroid doses (0, 0.25, 0.50, 0.75, 1.0 mg L^{-1}) in the presence and absence of humic acids during four sampling periods (60, 90, 120 and 150 days after transplanting), with five replicates for each treatment. The experimental unit was composed of 4 individually grown plantlets.

The BRS Vitória pineapple cultivar plantlets, propagated *in vitro*, were supplied by the BIOMUDAS biofactory, located at Venda Nova dos Imigrantes – ES, Brazil. The plantlets were transplanted into 200-cell polystyrene trays, pre-filled with the commercial Basaplant® Hortaliças substrate mixture, sieved through a 2-mm mesh and 20% of expanded vermiculite was added. The trays were then placed in a greenhouse, characterizing the beginning of the acclimatization process.

BIOBRAS-16® (spiro-static analog of castasterone - (25R) -2α, 3α-dihydroxy-5α-spirostan-6-one) was used as a brassinosteroid source, applied two days after plantlet transplanting and at 30 day intervals throughout the experimental period. The foliar route was used for the growth regulator applications, by means of solution sprays of each respective treatment (0.1% of Tween 20 was added as a surfactant).

The organic soil conditioner Agrolmin HF® was used as a humic acid source. Table 1 displays the chemical characteristics of this product. Humic acid applications were carried out at 15 days after plantlet transplanting and at 15-day intervals during the experiment, at 15 mmol L^{-1} of C, applied directly to the plantlet substrate, with the aid of an automatic pipette (totaling nine applications). Deionized water was applied in the control treatment.

Each week, 2 mL of the nutrient solution, with the following composition, in mg L^{-1}, were applied, per plantlet: $N(NO_3^-)$ = 112; $N(NH_4^+)$ = 3.5; P = 7.74; K = 156.4; Ca = 80; Mg = 24.3; S = 32.00; Cl =1.77; Mn = 0.55; Zn = 0.13; Cu = 0.03; Mo = 0.05; B = 0.27; Fe = 2.23, at pH = 5.5. Every 15 days, 2 mL of a urea solution at 1 g L^{-1} were also applied per plantlet.

The following characteristics were evaluated in the pineapple plantlets in each sampling period; foliar area was carried out on a bench leaf area measurement equipment (model LI-3100 LICOR, Lincoln, NE, USA), using a graduated ruler, with plantlet leaves grouped upwards, measuring from the base to the end of the larger leaf; root and aerial dry mass, after drying in a greenhouse under forced ventilation at 70 °C for 72 hours and finally, nitrogen, phosphorus, potassium, calcium and magnesium contents.

To determine the nutrient content of the aerial portions, the samples were ground and an aliquot from each treatment was weighed and sulfur digested, for nitrogen content determinations, while another was nitro-perchloric digested, for phosphorus, potassium, calcium and magnesium content determinations. Organic nitrogen was determined by the Nessler method (Jackson, 1965). Phosphorus was determined colorimetrically by the molybdate method, calcium and magnesium by atomic absorption spectrometry and potassium by atomic emission spectrophotometry (Malavolta et al., 1997).

The data were submitted to an analysis of variance by the F test, and the means obtained for the humic acid factor were compared by Tukey's test (p = 0.05), while the means for the brassinosteroid doses and sampling times were submitted to a regression analysis ($p < 0.05$).

RESULTS AND DISCUSSION

Table 2 displays the data obtained by the analysis of variance with the F values of each evaluated variable. The humic acid applications in pineapple plantlets led to a 6.2% increase in plantlet length at brassinosteroid dose

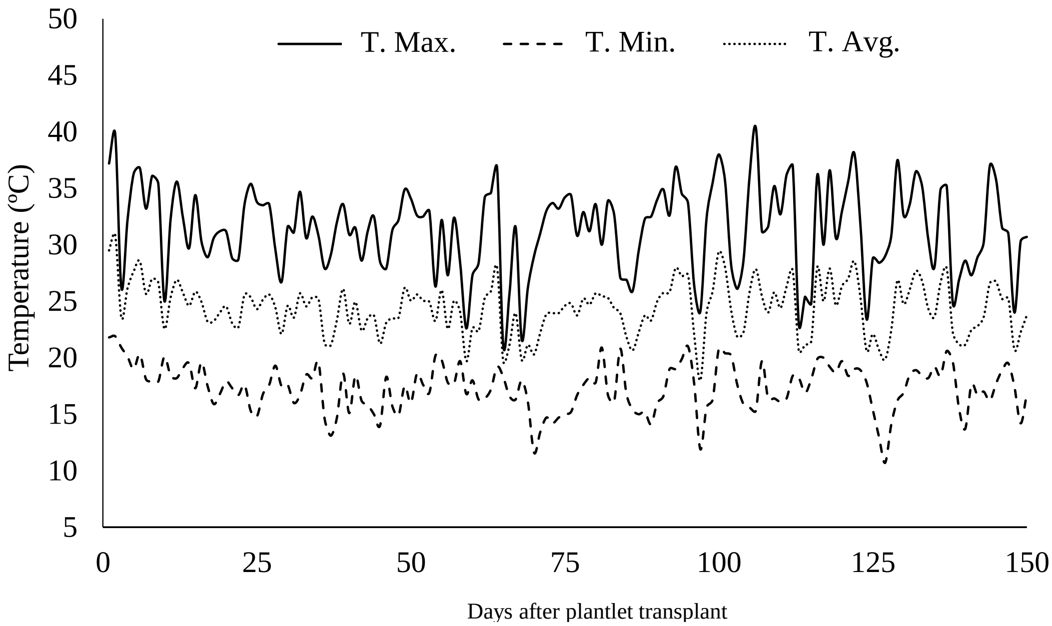

Figure 1. Minimum (T. Min.), average (T. Avg.) and maximum (T. Max.) temperatures recorded in the greenhouse during the experiment, at Campos dos Goytacazes - RJ.

Table 1. Chemical characteristics of Agrolmin® humic acid-based soil conditioner.

C_{org} total	Soluble N	K_2O	Zn	B	Density
(g L^{-1})					
108	16.2	16.2	3.78	2.16	1.08

Lot: 400.014, Production: 02/25/11.
Source of raw material: Potassium Hydroxide, Urea, Peat, Zinc Sulphate, Boric Acid and Water.

0 (Table 3). Plantlet length is an important biometric characteristic in the acclimatization process, indicated by several biofactories for the determination of the end point of this phase. Normally, plantlets are commercialized for acclimatization when they are 6 to 7 cm long (Berilli et al., 2011).

During the acclimatization phase, pineapple plantlets undergo structural and physiological adjustments, altering their metabolism from mixotrophic to autotrophic. This stage is very time-consuming regarding productive crop cycles, especially due to the growth slowness of both the aerial portion and the root system. Several factors can influence this process and impact plantlet production and adaptation (Barboza et al., 2006).

Both the number of leaves and plantlet length are used to evaluate vegetative growth during the acclimatization phase, mainly because they are correlated to foliar area and the dry mass of the aerial portion. When pineapples grow in favorable climatic conditions and in the field, they emit on average one leaf per week until inflorescence production (Giacomelli, 1982).

The application of humic acids independent of brassinosteroid dose resulted in a 2.6% increase in the number of plantlet leaves (Table 3), in agreement with the results reported by Baldotto et al. (2009), who also evaluated 'BRS Vitória' pineapple plantlets during the acclimatization phase and verified increases in growth rates and in the aerial parts after treatment with humic acids at 15 mmol L^{-1} of carbon. Application of Humic acid applications may have promoted higher plant growth by positively influencing ion transport, facilitating nutrient uptake (Nardi et al., 2002).

As mentioned previously, although plantlet length is one of the most applied characteristics to estimate the end of the acclimatization period, foliar area could also be used for this purpose according to Moreira et al. (2006), since higher foliar area values can provide greater photosynthetic area for the production of organic matter.

Regarding plantlet leaf area in response to brassinosteroid doses as a function of acclimatization periods, the increase rates (regression equations) of the plantlets that received growth regulator applications were higher compared to the treatment that received the lowest dose (Table 4).

The aerial dry mass production of pineapple plantlets was not influenced by humic acid and brassinosteroid applications.

Table 2. Summary of the variance analysis of the variables: number of leaves, length, leaf area, aerial dry mass, root dry mass, nitrogen, phosphorus, potassium, calcium and magnesium contents of 'BRS Vitória' pineapple plantlets with the application of five brassinosteroid doses (BRs) with or without the application of humic acids (HA) evaluated at four sampling times.

Variation causes	F values				
	Number of leaves	Plantlet length (cm)	Aerial dry mass (mg)	Root dry mass (mg)	Foliar area (cm²)
BRs	1.09^{ns}	2.52**	1.63^{ns}	5.86**	3.00**
AH	6.20**	0.87^{ns}	0.01^{ns}	32.00**	2.45^{ns}
Period	719.97**	856.01**	581.23**	360.42**	919.79**
BRs*AH	0.71^{ns}	3.08**	0.86^{ns}	6.69**	2.17^{ns}
BRs*Period	0.76^{ns}	1.94**	0.40^{ns}	3.42**	6.70**
AH* Period	1.68^{ns}	0.41^{ns}	1.18^{ns}	9.32**	1.35^{ns}
BRs*AH* Period	1.39^{ns}	1.06^{ns}	0.96^{ns}	3.16**	1.72^{ns}
General means	12.67	7.26	286.05	51.8	28.10
CV (%)	7.11	7.22	19.41	23.66	11.88

Variation causes	F values				
	Nitrogen	Phosphorous	Potassium	Calcium	Magnesium
			(g kg^{-1})		
BRs	2.98**	5.05**	1.24^{ns}	0.43^{ns}	7.68**
AH	0.67^{ns}	10.96**	2.16^{ns}	0.14^{ns}	0.52^{ns}
Period	424.89**	66.34**	84.55**	31.00**	16.18**
BRs* AH	1.53^{ns}	2.65**	4.44**	0.89^{ns}	1.25^{ns}
BRs* Period	2.10**	3.00**	1.55^{ns}	2.56**	1.59^{ns}
AH* Period	0.32^{ns}	1.93^{ns}	0.26^{ns}	9.88**	3.08**
BRs*AH* Period	1.38^{ns}	2.13**	3.24**	0.94^{ns}	0.93^{ns}
General means	13.04	1.77	40.2	8.79	3.17
CV (%)	13.16	15.19	8.78	7.89	13.05

^{ns} non-significant,* significant at p≤ 0.05 by the F test and ** significant at p ≤ 0.01 by the F test.

Table 3. Length and number of leaves of 'BRS Vitória' pineapple plantlets as a function of the application of five brassinosteroid doses acclimatized in a greenhouse.

Application of humic acids	Plantlet length (cm)					Number of leaves
	Brassinosteroid doses (mg L^{-1})					Mean of all brassinosteroid doses
	0	0.25	0.50	0.75	1.00	
With	7.49^{a}	7.00^{a}	7.38^{a}	7.04^{a}	7.20^{a}	12.83^{a}
Without	7.05^{b}	7.19^{a}	7.50^{a}	7.31^{a}	7.40^{a}	12.51^{b}

Means followed by the same letter in the columns do not differ by the Tukey test (p ≤ 0.05).

Their growth was adjusted with a quadratic equation ($\hat{y} = 0.0004x^2 + 4.7803x - 220.88$ $R^2 = 0.99$ **) as a function of the acclimatization period. However, Catunda et al. (2008) reported an 2.8-fold higher increase in dry mass accumulation in 'BRS Imperial' pineapples treated with 0.1 mg L^{-1} brassinosteroids compared to controls, while Freitas et al. (2012), when evaluating the effect of brassinosteroid doses ranging from 0 to 1 mg L^{-1} on the growth of 'Smooth Cayenne' pineapple plantlets through stem sectioning, found that the estimated dose of 0.68 mg L^{-1} provided higher aerial dry mass.

Plantlet root systems were significantly altered by humic acid applications, with a 63% increase in root dry matter at 150 days after transplanting (Table 5).

Micropropagated plantlets with greater root mass may be more vigorous and can present better adaptations to the acclimation and field planting phases, since the more developed the root system, the greater the nutrient absorption and, consequently, the greater the plant growth. The results of this study corroborate those reported by Baldotto et al. (2010) that observed positive effects on micropropagated pineapple plantlets when humic acids were applied.

The main effects of humic acids on growth are responses to the stimuli this substance causes, similar to the effects of the

Table 4. Increase of leaf area, nitrogen and calcium content in 'BRS Vitória' pineapple plantlets as a function of brassinosteroid (BRs) application at 150 days after the beginning of acclimatization.

BRs (mg L^{-1})	Equation	R^2	Foliar area (cm^2)	Increment in relation to BRS dose 0 (%)
0	$\hat{y} = 0.312x – 5.781$	0.99**	41.02	-
0.25	$\hat{y} = 0.386x – 11.67$	0.84**	46.23	12.7
0.50	$\hat{y} = 0.363x – 8.96$	0.95**	45.49	10.9
0.75	$\hat{y} = 0.344x – 8.134$	0.93**	43.46	5.9
1.00	$\hat{y} = 0.354x – 8.827$	0.97**	44.27	7.9

BRs (mg L^{-1})	Equation	R^2	Nitrogen content (g kg^{-1})	Increment in relation to BRS dose 0 (%)
0	$\hat{y} = -0.1351x + 27.50$	0.84**	7.24	-
0.25	$\hat{y}= -0.1306x + 27.25$	0.85**	7.66	5.8
0.50	$\hat{y}= -0.1033x + 23.13$	0.89**	7.63	5.4
0.75	$\hat{y} = -0.1249x + 26.10$	0.86**	7.36	1.7
1.00	$\hat{y} = -0.1085x + 24.50$	0.88**	8.23	13.7

BRs (mg L^{-1})	Equation	R^2	Calcium content (g kg^{-1})	Increment in relation to BRS dose 0 (%)
0	$\hat{y}= -0.0003x^2 + 0.07x + 5.11$	0.60**	8.41	-
0.25	$\hat{y}= -0.0003x^2 + 0.07x + 5.07$	0.93**	8.36	-0.6
0.50	$\bar{y} = 8.80$	-	8.80	4.6
0.75	$\bar{y} = 8.78$	-	8.78	4.4
1.00	$\hat{y} = 0.0131x + 7.33$	0.59**	9.30	10.6

* Significant at $p \leq 0.05$ by the F test and ** significant at $p \leq 0.01$ by the F test.

phytohormone auxin, which stimulates plasma membrane H-ATPase electrogenic pump activity (Hager et al., 1991), causing cell expansion and activation of secondary transporters in cell membranes (Sondergaard et al., 2004).

When humic acids were applied with 0.25, 0.50, 0.75 and 1.00 mg L^{-1} brassinosteroids, 16, 19, 23 and 39% reduction in root dry mass were observed, respectively, when compared to treatments with humic acid and 0 mg L^{-1} of brassinosteroids (Table 5). These results suggest that the biostimulating effect of humic acids on the root system can be reduced with brassinosteroid applications. Roddick et al. (1993) reported that continued brassinosteroid applications promote root growth inhibition.

Pineapple development is dependent on nutritional status, and when plantlets are cultivated under elemental deficiencies, their growth rate and development can be reduced and may even interfere in later developmental stages (Silva et al., 2016). However, little is known about adequate nutrient content during the pineapple acclimatization phase.

Increasing brassinosteroids doses caused a 13.7% increase in foliar nitrogen levels, when comparing the plantlets that received the lowest dose of this growth regulator to those that received the highest dose (Table 4). It is noteworthy that, regardless of growth regulator dose, nitrogen contents were reduced in 63, 61, 55, 69 and 54% for the 0, 0.25, 0.50, 0.75 and 1.0 mg L^{-1} brassinosteroid doses, respectively, from 60 to 150 acclimatization days (Table 4). However, this effect was lower for the 0.50 and 1.00 mg L^{-1} doses.

These results are in agreement with those reported by Freitas et al. (2012), who verified that brassinosteroid doses exhibited a significant effect on nitrogen content, with an increasing linear behavior, with 1.0 mg L^{-1} providing an 11.1% increase in the nutritional content of pineapple plantlets when compared to the controls. Freitas et al. (2015), when working with 'Cleopatra' mandarin plantlets, reported that 1.0 mg L^{-1} brassinosteroids associated with arbuscular mycorrhizal fungi led to a 15.4% increase in the aerial dry mass nitrogen content.

Diniz et al. (1999) evaluated *in vitro* macronutrient absorption by banana explants, and reported that nitrogen content was higher during the first 10 days, decreasing as a function of culture time. According to the authors, this decrease in nitrogen content in tissues may occur as a function of the dilution effect, due to higher dry mass production. This observation corroborates the results obtained herein, where higher nitrogen content was also observed in aerial portions at 60 days after transplanting, followed by a decrease as a function of time (Table 4).

The highest phosphorus levels were recorded in plantlets that received humic acids alongside brassinosteroids, while the opposite was observed for potassium, where the joint application of humic acids and brassinosteroids led to lower potassium contents when compared to plantlets treated with humic acids only (Table 5).

The 1.00 mg L^{-1} brassinosteroids dose led to a 10.6% increase in leaf calcium content in pineapple plantlets at 150 days of acclimatization (Table 4), while magnesium

Table 5. Increment of root dry mass and phosphorus and potassium contents in 'BRS Vitória' pineapple plantlets as a function of brassinosteroid (BRs) and humic acid (HA) applications at 150 days after acclimatization.

HA	BRs (mg L^{-1})	Equation	R^2	Dry root mass (mg)	Increment in relation to BRS dose 0 (%)
	0	$\hat{y} = 0.7048x - 31.11$	0.94**	74.6	-
	0.25	$\hat{y} = 0.663x - 25.34$	0.88**	74.1	-1
Without	0.50	$\hat{y} = 0.5551x – 13.04$	0.87**	70.2	-6
	0.75	$\hat{y} = 0.6534x - 21.27$	0.91**	76.7	3
	1.00	$\hat{y} = 0.4838x – 4.965$	0.78*	67.6	-9
	0	$\hat{y} = 1.1913x - 56.71$	0.97**	121.9	63
	0.25	$\hat{y} = 1.0161x - 49.99$	0.85**	102.0	37
With	0.50	$\hat{y} = 0.8735x - 31.93$	0.83**	99.01	33
	0.75	$\hat{y} = 0.8815x - 38.43$	0.81**	93.8	26
	1.00	$\hat{y} = 0.591x – 14.03$	0.86**	74.6	0

HA	BRs (mg L^{-1})	Equation	R^2	Phosphorous content (g kg^{-1})	Increment in relation to BRS dose 0 (%)
	0	$\hat{y} = 0.0002x^2 - 0.05x + 3.97$	0.55**	1.03	-
	0.25	$\hat{y} = 0.0001x^2 - 0.019x + 2.32$	0.68**	1.72	67
Without	0.50	$\hat{y} = 0.0002x^2 - 0.048x + 4.03$	0.67**	1.30	26
	0.75	$\hat{y} = 0.0001x^2 - 0.02x + 2.44$	0.69**	1.69	64
	1.00	$\hat{y} = 0.0002x^2 - 0.043x + 3.62$	0.83**	1.74	69
	0	$\hat{y} = 0.0001x^2 - 0.019x + 2.48$	0.73**	1.86	81
	0.25	$\hat{y} = 0.0004x^2 - 0.070x + 4.51$	0.99**	3.00	191
With	0.50	$\hat{y} = 0.0003x^2 - 0.055x + 4.3$	0.73**	2.85	177
	0.75	$\hat{y} = 0.0002x^2 - 0.05x + 3.78$	0.74**	2.62	154
	1.00	$\hat{y} = 0.0002x^2 - 0.035x + 3.20$	0.73**	2.30	123

HA	BRs (mg L^{-1})	Equation	R^2	Potassium content (g kg^{-1})	Increment in relation to BRS dose 0 (%)
	0	$\hat{y} = -0.1831x + 57.49$	0.95**	30.0	-
	0.25	$\hat{y} = -0.1345x + 55.34$	0.97**	35.3	18
Without	0.50	$\hat{y} = -0.0752x + 47.99$	0.47**	36.7	22
	0.75	$\hat{y} = -0.0821x + 48.81$	0.77**	36.5	22
	1.00	$\hat{y} = -0.0791x + 48.88$	0.52**	37.0	23
	0	$\hat{y} = -0.0579x + 48.49$	0.96**	39.8	33
	0.25	$\hat{y} = -0.0794x + 46.25$	0.61**	34.3	14
With	0.50	$\hat{y} = -0.1506x + 55.92$	0.87**	33.3	11
	0.75	$\hat{y} = -0.1651x + 55.75$	0.78**	30.9	3
	1.00	$\hat{y} = -0.1164x + 53.71$	0.79**	36.3	21

* Significant at $p \leq 0.05$ by the F test.

content was observed as a function of brassinosteroid application, by means of the following equation: $\hat{y} = -0.368x + 3.35$ $R^2 = 0.65$ **, where contents decreased linearly, with a 11% reduction when a higher dose of the growth regulator was applied compared to the 0 mg L^{-1} brassinosteroid dose.

The highest calcium and magnesium contents were observed when humic acids were applied (Figure 2). Cordeiro et al. (2010) cite that, in addition to the biostimulating effect on plant growth, humic acids can provide nutrients to plants through their mineralization.

The results of this study point out the potential of the use of products based on humic acids and brassinosteroids in decreasing pineapple propagation periods. However, further studies on these treatments regarding acclimatization and on the effective behavior of the plantlets after transplanting them to beds during the acclimatization phase should be carried out.

Conclusions

The application of humic acids via substrate in 'BRS Vitória' pineapple plantlets favors the growth of both the aerial portion and root system, while also increasing phosphorus, potassium, calcium and magnesium

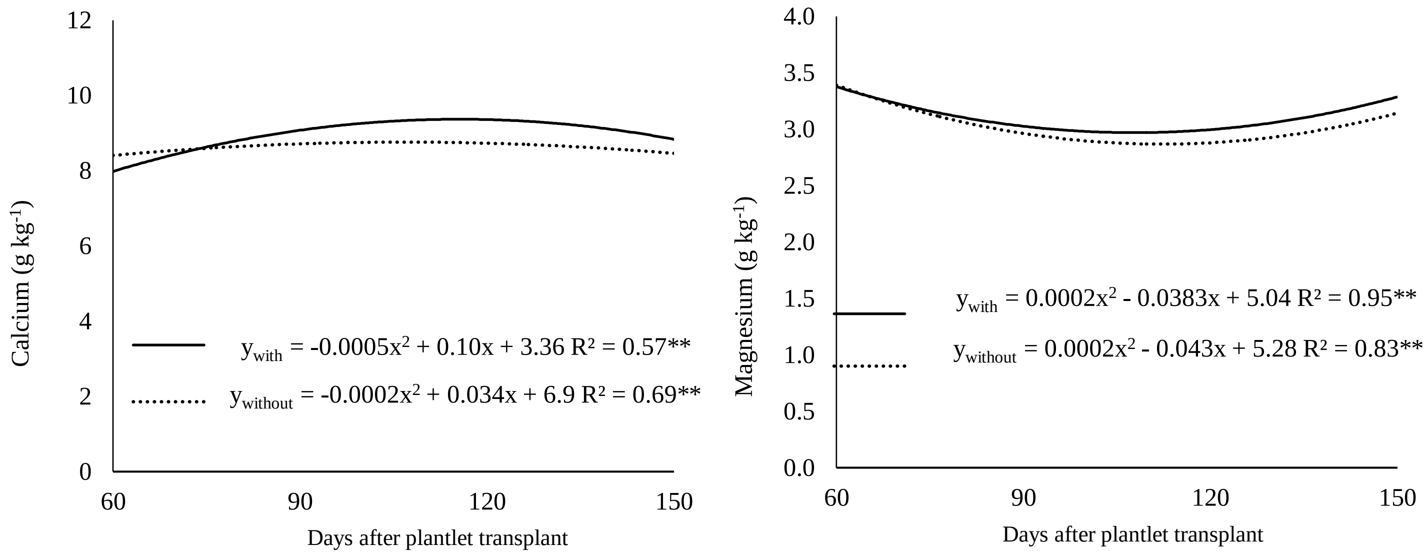

Figure 2. Calcium and magnesium contents of 'BRS Vitória' pineapple plantlet grown with or without humic acids as a function of four periods (60, 90, 120 and 150) after transplanting. * Significant at $p \leq 0.05$ for the F test.

contents, optimizing the acclimatization period. Therefore, this substance can be considered an ally in reducing the acclimatization period.

The application of brassinosteroids at 0.25 to 1.00 mg L^{-1} doses, allied or not to the application of humic acids, promotes a depression effect on the root systems of BRS Vitória pineapple plantlets. On the other hand, the application of this substance increases leaf area and nitrogen, phosphorus, potassium and calcium contents.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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