Photoperiod and growth regulators on *in vitro* shoot induction in *Heliconia* latispatha[1]

ANTONIO ANDERSON DE JESUS RODRIGUES[2], EDER DE OLIVEIRA SANTOS[3]
and ANA CRISTINA PORTUGAL PINTO DE CARVALHO[4]*

ABSTRACT

Considering the growing economic importance of tropical flowers and the advantages of techniques applied to the *in vitro* cultivation of these plants, it is necessary to carry out studies to evaluate growth in species such as *Heliconia latispatha*. The aim of this study therefore, was to evaluate *in vitro* shoot induction for different concentrations of BAP and NAA and as a function of the photoperiod. Explants from zygotic embryos were inoculated in MS medium containing different concentrations of BAP (0.0, 0.5, 1.0, 1.5, 2.0 or 2.5 mg L^{-1}), with the cultures kept in a growth room at a temperature of 24.0 ± 2.0° C, under a photoperiod of 12 and 16 hours of light and a light intensity of 30 µmol m^{-2} s^{-1}. At 21, 28, 35, 42 and 49 days after inoculation, the number of shoots per explant was evaluated. The treatment at the BAP concentration that gave the best multiplication rate (2.5 mg L^{-1}) was set, and was tested in a further trial with different concentrations of NAA (0.0, 0.2, 0.4, 0 6, 0.8 or 1.0 mg L^{-1}) under the same conditions as the previous experiment. The experimental designs were completely randomised, with five replications, and analysed in a 6 x 2 factorial. The data were submitted to analysis of variance and regression. No significant differences were seen in relation to the photoperiod or its interaction with the cytokinin and auxin under test. Multiplication was greater in the presence of 2.5 mg L^{-1} BAP, which gave a rate of 1.25 shoots/explant at 49 days of *in vitro* culture. The association of this BAP dosage with 1.0 mg L^{-1} NAA was even more efficient, producing 1.83 shoots per explant at 30 days of growth. The use of BAP together with NAA is beneficial to the induction of shoots in *H. latispatha*.

Keywords: Heliconiaceae, 6-benzylaminopurine, naphthaleneacetic acid, tissue culture.

RESUMO

Indução de brotações *in vitro* de *Heliconia latispatha* em função do fotoperíodo e reguladores de crescimento

Considerando a crescente importância econômica das flores tropicais e as vantagens das técnicas aplicadas ao cultivo *in vitro* para estas plantas, torna-se necessária a realização de estudos que avaliem a propagação de espécies como a *Heliconia latispatha*. Diante do exposto, o objetivo do trabalho foi avaliar a indução de brotações *in vitro* em função de diferentes concentrações de BAP e ANA em função de fotoperíodos. Os explantes, oriundos de embriões zigóticos, foram inoculados em meio MS contendo diferentes concentrações de BAP (0,0; 0,5; 1,0; 1,5; 2,0 ou 2,5 mg L^{-1}), mantendo-se as culturas em sala de crescimento com temperatura de 24,0 ± 2,0 ºC sob fotoperíodo de 12 e 16 horas de luz e intensidade luminosa de 30 µmol m^{-2} s^{-1}. Aos 21, 28, 35, 42 e 49 dias após a inoculação, foi avaliado o número de brotos obtidos por explante. O tratamento com a concentração de BAP que proporcionou melhor taxa de multiplicação (2,5 mg L^{-1}) foi fixado e, em outro ensaio foi testado com diferentes concentrações de ANA (0,0; 0,2; 0,4; 0,6; 0,8 ou 1,0 mg L^{-1}) nas mesmas condições do experimento anterior. O delineamento experimental dos experimentos foi o inteiramente casualizado, com cinco repetições, analisado em esquema fatorial 6 x 2. Os dados foram submetidos à análise de variância e à regressão. Não foram observadas diferenças significativas com relação ao fotoperíodo nem sua interação com a citocinina ou auxina testada. A taxa de multiplicação foi maior na presença de 2,5 mg L^{-1} de BAP, que proporcionou uma taxa de multiplicação de 1,25 brotos/explante aos 49 dias de cultivo *in vitro*. A associação dessa dosagem de BAP com 1,0 mg L^{-1} de ANA foi ainda mais eficiente produzindo 1,83 brotos por explante em 30 dias de cultivo. O uso associado de BAP e ANA é benéfico para a indução *in vitro* de brotações de *H. latispatha.*

Palavras-chave: Heliconiaceae, 6-benzilaminopurina, ácido naftaleno acético, cultura de tecidos.

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[2] Universidade Federal do Ceará (UFC), Departamento de Fitotecnia, Fortaleza-CE, Brazil.

[3] Universidade Federal do Ceará (UFC), Departamento de Ciências do Solo, Fortaleza-CE, Brazil.

[4] Embrapa Agroindústria Tropical (EMBRAPA CNPAT), Laboratório de Cultura de Tecidos Vegetais, Fortaleza-CE, Brazil. *Corresponding author: cristina.carvalho@embrapa.br

1. INTRODUCTION

Over the past few years, the Brazilian market for flowers and ornamental plants has been growing dynamically. In 2013, floriculture in Brazil had a turnover of approximately BRL 1.49 billion, an increase of 57.56% (JUNQUEIRA and PEETZ, 2014).

Considered profitable, the cultivation of tropical flowers has been on the increase and is now regarded as a viable alternative in small rural areas (COELHO et al., 2016). Heliconia stands out among tropical flowers due to its high consumer acceptance, a result of the shape and intense colouring and exoticism of the inflorescence, and to its high post-harvest durability (LIMA et al., 2016).

Castro et al. (2007), evaluating 30 genotypes of heliconia for suitability as cut flowers, included the species *Heliconia lathispatha* among those classified as moderately suitable. Among its features, the inflorescence of the species was classified as having a light orange coloration, with flowering spread over the summer months (January to March) and the beginning of autumn (April), and flowering intervals of 90 to 120 days. It should be noted that, of the species of heliconia classified as moderately suitable for cut flowers, *Heliconia latispatha* is still little cultivated or traded in the domestic market.

In vitro techniques of propagation of flowers and ornamental plants are being increasingly used commercially, as the floriculture sector demands a great number of uniform plants of high genetic and phytosanitary quality throughout the year (CARVALHO et al., 2013).

In the tissue culture laboratory, luminosity in the growth rooms is an important factor, as this has an influence on the proper development of the plants *in vitro* (BARRUETO CID and TEIXEIRA, 2010). In micropropagation, the most widely used photoperiods are 12 hours of light / 12 hours of darkness or 16 hours light / 8 hours of darkness (RIBEIRO et al., 2009; SILVA et al., 2012; GONZALEZ and CUEVA, 2014; SANTOS et al., 2015; SANTOS et al., 2016).

In most of the studies carried out with different species of heliconia, the photoperiod used was 16 hours (NATHAN et al., 1992; DINIZ et al., 2004; RODRIGUES et al., 2006; GUZMAN et al., 2009; ALARCÓN et al., 2011), however the authors did not evaluate the effect of photoperiod on the *in vitro* micropropagation of the species (BRAGA et al., 2009).

In addition to the luminosity, another factor that can influence *in vitro* cultivation is the use of growth regulators. Variations in the composition of growth regulators have been widely tested due to species and type of explant responding differently to the conditions of *in vitro* cultivation (LEMOS, 2010). Marulanda-Ángel et al. (2011) tested six combinations of 6-benzylaminopurine (BAP) in the *in vitro* cultivation of *Heliconia bihai* (L.) cv. Lobster Salmón, and achieved a higher multiplication rate for the species when they combined the cytokinin BAP with indoleacetic acid (IAA).

A combination of the regulators influenced shoot production in relation to size and vigour. Bearing in mind that the *in vitro* cultivation of *Heliconia latispatha* has to date not been reported in the literature, it becomes necessary to establish responsive micropropagation protocols for the production of plantlets of high phytosanitary and genetic quality on a large scale, in order to meet the demands of commercial production. The aim of this study therefore was to evaluate the influence of the growth regulators BAP and NAA and of photoperiod on the *in vitro* shoot induction of *Heliconia latispatha* Bentham cv. Orange Gyro.

2. MATERIAL AND METHODS

The explants were obtained from cultures previously established *in vitro* from zygotic embryos and multiplied by successive subcultures, under a photoperiod of 12 and 16 hours, in a basic MS culture medium (MURASHIGE and SKOOG, 1962) supplemented with 30 g L^{-1} sucrose and solidified with 5.5 g L^{-1} agar.

The shoots used as explants had an average height of 2.5 cm, a diameter of 5.5 mm, from 3 to 4 leaves and were without roots. The pH of the culture medium was adjusted to 5.8 and the medium autoclaved at 121°C and 1 atm for 20 minutes. The culture medium was supplemented with six concentrations of BAP (0.0, 0.5, 1.0, 1.5, 2.0 or 2.5 mg L^{-1}), to give a total of 12 treatments.

Glass jars were used, 10 cm in height and 6.3 cm in internal diameter, having a volume of 220 mL and with a polypropylene screw cap; these contained 30 mL of culture medium. The explants were inoculated into the nutrient medium under aseptic conditions in a laminar flow cabinet.

The experimental design was completely randomised with five replications per treatment, and analysed in a 2 x 6 factorial (two photoperiods x six concentrations of BAP), giving a total of 60 plots. Each replication consisted of one jar containing four explants. The cultures were kept in two growth rooms, at photoperiods of 12 and 16 hours according to treatment, both rooms at a temperature of 24 ± 2°C and a light intensity of 30 μmol m^{-2} s^{-1}.

At 21, 28, 35, 42 and 49 days after inoculation of the explants *in vitro*, an evaluation was made of the number of shoots per explant.

The best concentration of BAP was 2.5 mg L^{-1}, which was then combined with different concentrations of naphthaleneacetic acid (NAA) (0.0, 0.2, 0.4, 0.6, 0.8 or 1.0 mg L^{-1}) at two photoperiods (12 and 16 hours). Thirty days after inoculation *in vitro* of the explants, the number of shoots produced by each explant was evaluated.

The data were submitted to analysis of variance and regression analysis using the Sisvar statistical software (FERREIRA, 2014).

3. RESULTS AND DISCUSSION

No difference was seen in multiplication rate for the photoperiods used. This could be due to the species having adapted through evolution to the high luminosity, a characteristic of the tropical regions where they occur naturally. According to Schock et al. (2014), a dependence on light in the physiological processes of plants reflects in their maintenance of homeostasis. As a result, most plants

develop the ability to acclimatise to variations of light and temperature (SEARLE et al., 2011), adjusting their photosynthetic apparatus for the proper absorption, transfer and use of the electromagnetic energy available in their environment (GONÇALVES et al., 2010).

In the present study, cool-white fluorescent lamps were used, which regardless of the period of light exposure may have contributed to the lack of variation in the multiplication rate of the species, being of lower quality compared to exposure to natural, solar light.

The photoperiod of 12 hours proved to be advantageous since it allows a reduction in electricity costs, thereby minimising the resources required to produce plantlets of *H. latispatha*, and resulting in a greater proportion of micropropagated plantlets.

It is important to note that for the micropropagation of heliconia no information is found in the literature about the performance of cultures under a photoperiod of 12 hours. The research work has been carried out exclusively under a photoperiod of 16 hours of light, generating good results, such as the increase in the number of shoots in *Heliconia stricta* Huber in a medium with 2.0 mg L^{-1} BAP (DINIZ et al., 2004); the study of somaclonal variation to promote such features as the reddish tone of the pseudostem in *Heliconia bihai* cv. Lobster Claw, that appears in the form of an equidistant spiral giving an exotic look to the flower stem (RODRIGUES et al., 2006); the *in vitro* regeneration of *Heliconia psittacorum* (GUZMÁN et al., 2009); the propagation of *Heliconia curtispatha* P (ALARCÓN et al., 2011); and the propagation of *Heliconia psittacorum* (NATHAN et al., 1992), among others.

In all five of the evaluations made, a difference was found for *H. latispatha* in the number of shoots produced per explant for the culture media under test. As for the effect of the different concentrations of BAP under test, in the regression analysis the best-suited mathematical model to the response curve in the first two evaluations, at 21 and 28 days, was linear, with values for the coefficient of determination shown in Table 1. With respect to the other three evaluations carried out at 35, 42 and 49 days, the most appropriate regression analysis adopted for the response curve was quadratic.

Table 1. Equations and coefficients of determination in shoot induction from explants of *Heliconia latispatha* Bentham cv. Orange Gyro in MS culture medium, for BAP concentrations, at 21, 28, 35, 42, and 49 days of *in vitro* cultivation.

Days of subcultivation	Equation	Coefficient of determination (R^2)
21	$y = 0.1643x + 0.7167$	0.8542
28	$y = 0.2236x + 0.6058$	0.8561
35	$y = 0.0787x^2 - 0.2749x + 1.3238$	0.9130
42	$y = 0.0850x^2 - 0.2450x + 1.2763$	0.9451
49	$y = 0.0967x^2 - 0.2655x + 1.3093$	0.9502

The initial linear increase in the multiplication rate for the first two evaluations (at 21 and 28 days) can be explained by the rapid early development of the explants, derived from a process of direct organogenesis, during the first weeks of *in vitro* growth and formation of the first shoots; with time this growth phase tends to display a typical stabilising behaviour, a characteristic tendency of the quadratic polynomial model obtained in the last three evaluations, at 35, 42 and 49 days of *in vitro* growth (Figure 1).

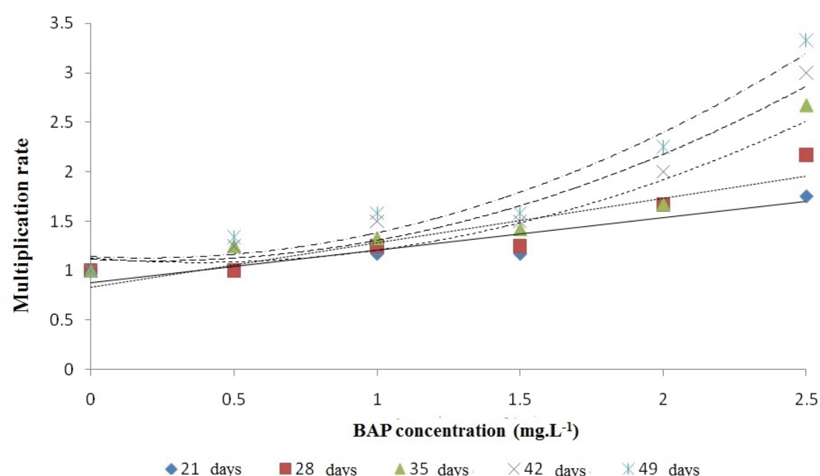

Figure 1. Response curve for the number of shoots per explant of *Heliconia latispatha* Bentham cv. Orange Gyro in MS culture medium, for BAP concentrations, at 21, 28, 35, 42 and 49 days of *in vitro* cultivation.

These results indicate that, with the evaluations at 21 and 28 days, the multiplication rate shows steady growth for increases in the BAP concentration.

With the remaining three evaluations, subsequently carried out at 35, 42 and 49 days, it was found that it was not possible to determine the maximum multiplication rate with any of the quadratic polynomial regressions, since the equations had a minimum point respectively of 1.08, 1.10 and 1.13. This may demonstrate that the ideal concentration of cytokinin to obtain the best multiplication rate for the species in question has not yet been reached, and that concentrations greater than those employed in this work should be tested, with checks also being made as to whether other cytokinins would not be more effective for *in vitro* multiplication. However, concentrations greater than the optimal have effects that are unfavourable to shoot induction due to hormonal imbalance between the plant regulators present in the culture medium (MONTFORT et al., 2012). It should also be noted that the use of very high concentrations of cytokinins could encourage the occurrence of somaclonal variation *in vitro*, as described in *Heliconia bihai* cv. Lobster Claw by Rodrigues (2008).

In general, regardless of the period of evaluation, no shoot formation was found in the culture medium without the addition of BAP (Figure 2A). A similar result was obtained by Nathan et al. (1992), who found a lack of shoot proliferation in *Heliconia psittacorum* L. f. when BAP was removed from the culture medium. According to Colombo et al. (2010), with *in vitro* cultivation, the use and combination of growth regulators is necessary to compensate for deficiencies in the endogenous hormone levels of the shoots, since these are isolated from the mother plant.

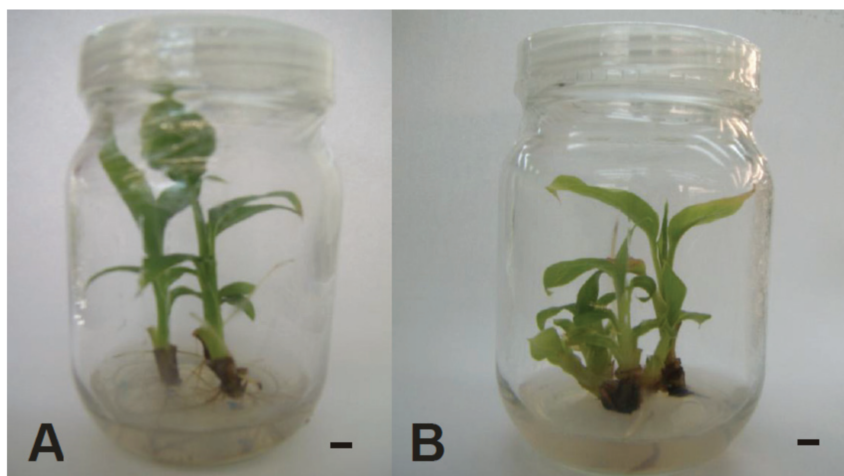

Figure 2. Plantlets of *Heliconia latispatha* Bentham cv. Orange Gyro, grown in MS culture medium, at 49 days of *in vitro* cultivation under a 12-hour photoperiod at the following concentrations: (A) MS without the addition of growth regulator; (B) MS + 2.5 mg L^{-1} BAP. Bars = 50 mm.

The addition of BAP to the culture medium favoured the emission of shoots in the explants. With regard to the most suitable concentration, the greatest estimated numerical values for multiplication rate were recorded in the medium containing 2.5 mg L^{-1}, with values of 1.20 at 42 days, and 1.25 at 49 days of *in vitro* growth.

Nathan et al. (1992) found higher rates of shoot proliferation in a medium containing 2.25 mg L^{-1} BAP at 45 days after the inoculation of explants of *Heliconia psittacorum* L. f. At that concentration of BAP, the authors recorded a multiplication rate of 5.0 shoots per explant. Diniz et al. (2004) found that the number of shoots was significantly higher in *Heliconia stricta* Huber with BAP present in the culture medium compared to the absence of this cytokinin. The concentration of 2.0 mg L^{-1} was the most efficient, promoting a multiplication rate of 2.4 shoots/explant at 40 days of growth.

Studying the *in vitro* propagation of *Heliconia standleyi* Macbride, Sosa-Rodriguez et al. (2009) reported values for multiplication rate of 2.3 shoots per explant in a culture medium containing 1.0 mg L^{-1} BAP, and 3.5 shoots per explant with the addition of 2.0 and 3.0 mg L^{-1} BAP after 30 days of *in vitro* growth. Comparing these values, it can be seen that the multiplication rates achieved with the medium containing 1.0, 2.0 and 2.5 mg L^{-1} BAP at 28 days were lower for the species under study, *H. latispatha*, where the recorded multiplication rates were respectively 0.83, 1.05 and 1.16.

Alarcon et al. (2011), in their research into the *in vitro* multiplication of *Heliconia curtispatha* under five doses of BAP (0.0, 0.5, 1.0, 2.0 and 4.0 mg L^{-1}) over 5 weeks (35 days), obtained a higher average (5.66 shoots/explant) with the treatment containing 2.0 mg L^{-1} BAP at 21 days (3rd week) of *in vitro* growth.

The beneficial action of BAP can be explained by the ability of plant tissue to metabolise natural hormones more quickly than synthetic hormones; it also has the lowest acquisition cost (BORGES JÚNIOR et al., 2004; ARAGON et al., 2011). Furthermore, BAP is one of the fastest absorbed of the cytokinins, acting quickly on plant metabolism and promoting the signalling of multiple shoot induction when grown in a culture medium (ROLLI et al., 2011; TAIZ and ZEIGER, 2013).

Under suitable concentrations, the presence of BAP in the culture medium triggers activation of an enzyme complex in the plants, responsible for stimulating the initial vegetative growth of the lateral buds by promoting cell division (MURAI, 2014).

Silva Júnior et al. (2015) achieved higher multiplication rates with *Etligera elatior* (Jack) R.M. Smith, for a concentration of 1.0 mg L^{-1} (5.5 shoots per explant) at 30 days, with the response curve displaying quadratic behaviour; a production of 3.8 shoots per explant was obtained at a concentration of 2.0 mg L^{-1} BAP. Furthermore, after 60 days of *in vitro* growth, the same study displayed linear growth behaviour and a multiplication rate of 5.8 shoots per explant at 2.0 mg L^{-1}, well above the present study. The same quadratic behaviour of the response curve was seen in *Etlingera elatior* cv. Porcelana, however a concentration of 0.5 mg L^{-1} BAP gave the best response for number of shoots, which at 30 days achieved a rate of 4.36 shoots per explant (SANTOS et al., 2016).

These differences in multiplication rate among the various species and cultivars studied may be a result of the interaction between the genotype employed and the physiological conditions of the explants at the time the shoots were collected for setting up the cultures, as well as the method of handling each experimental group, and the conditions in the growth rooms where the cultures were kept during the trials (DINIZ et al., 2004).

In order to observe an increase in multiplication rate, the treatment that produced the greatest number of shoots (2.5 mg L^{-1} BAP) was used with different concentrations of naphthaleneacetic acid.

In the evaluation, it was found that for the species of heliconia under study, the value for number of shoots produced per explant varied with the culture medium being tested. In the regression analysis of the effect of the different concentrations of NAA, the most appropriate mathematical model was linear, with a coefficient of determination of 0.8652.

For the number of shoots, differences were seen between concentrations of NAA. It was found that with the increase in NAA concentration there was an increase in the number of shoots, reaching an approximate multiplication rate of 1.83 shoots per explant at 30 days of *in vitro* growth (Figure 3).

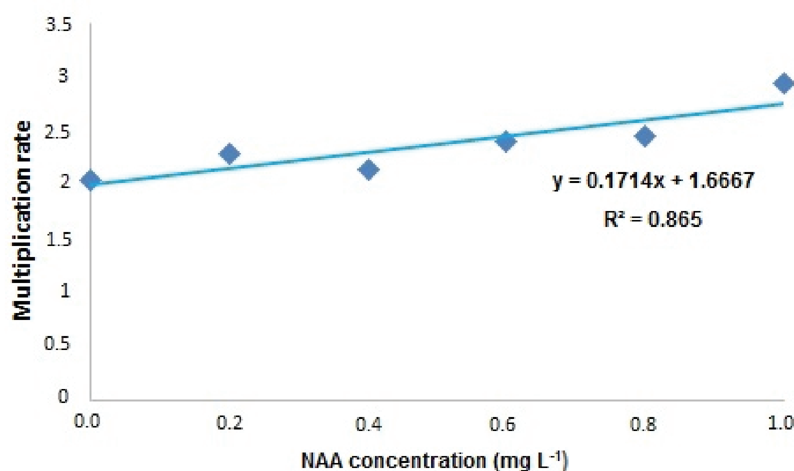

Figure 3. Response curve for the number of shoots per explant of *Heliconia latispatha* Bentham cv. Orange Gyro in MS culture medium containing 2.5 mg L^{-1} BAP combined with different NAA concentrations, at 30 days of *in vitro* cultivation.

It can be seen that using a combination of 2.5 mg L^{-1} BAP and 1.0 mg L^{-1} NAA gave a higher average compared to the treatment containing only 2.5 mg L^{-1} BAP of the previous experiment.

The beneficial effect of combining cytokinin and auxin was also seen by Marulanda-Ángel et al. (2011) who, studying the *in vitro* multiplication of *Heliconia bihai* (L.) cv. Lobster Salmón, obtained the best multiplication rate (3.0 shoots/explant) with a combination of 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} IAA at 28 days of *in vitro* growth.

Colombo et al. (2010), working with *Etlingera elatior* (Jack) R.M. Sm, obtained an average of 3.70 shoots/explant in a culture medium containing 3.37 mg L^{-1} NAA and 4.5 mg L^{-1} BAP after 90 days of growth.

Silva Júnior et al. (2015) obtained superior results when testing different concentrations of NAA and a fixed dose of BAP, where the rate of shoots displayed a response curve with increasing linear behaviour in *Etlingera elatior* var. Red Torch for increasing concentrations of auxin, culminating in a rate of 9.82 shoots/explant at 90 days of growth in a culture medium containing 3.0 mg L^{-1} BAP and the addition of 1.0 mg L^{-1} NAA.

The fact there was an increase in the number of shoots for increases in the concentration of auxin, shows the importance of establishing the proper ratio of auxin to cytokinin that appears to be beneficial to shoot production in the explant (LEMOS, 2010).

4. CONCLUSIONS

The presence of BAP in the culture medium promotes the emission of shoots in *Heliconia latispatha* Bentham cv. Orange Gyro. Due to economic factors, a photoperiod of 12 hours is recommended. The addition of NAA to the culture medium, associated with an established BAP dosage, favours an increase in the number of shoots grown *in vitro*, enabling plantlets production in *Heliconia latispatha* cv. Orange Gyro.

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