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A N I N T E R N A T I O N A L J O U R N A L

Crude Fermented Extract Containing Gibberellic Acid Produced by *Fusarium moniliforme* is an Alternative to Cost Reduction in Biofactories

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ABSTRACT

Nidularium procerum and Nidularium innocentii (Bromeliaceae) were cultivated in vitro on media supplemented with different sources and levels of GA_3 (gibberellic acid). These sources were the commercial powder (analytical degree) and fermented extract obtained by Fusarium moniliforme via solid state fermentation. The in vitro elongation and rooting of these plants were evaluated after 50 days of cultivation. The GA_3 present in the fermented extract possess the same effect of purified GA_3 (analytical degree) for the increase of the height of aerial part of shoots of N. innocentii, but not for the N. procerum being the GA_3 fermented extract in a lesser degree. The GA_3 fermented extract influences negatively the rooting in N. innocentii, while GA_3 analytical degree practically does not interfere in the rooting. On the other hand, in N. procerum, both the GA_3 sources reduce the root number and do not interfere in rooting percentage. GA_3 crude fermented extract is an alternative to reduce costs, however, its results can vary depending on the species and parameter evaluated. The fermented extract was stored at temperature during 260 days and its shelf life presented a suitable stability, maintaining 92% of its initial GA_3 amount.

Keywords: Shelf life, bromeliad, gibberellin, solid state fermentation, micropropagation, biofactory.

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INTRODUCTION

Plant tissue culture is the only methodology that can produce a large quantity of clonal plants in a short time with high phytosanitary quality [1]. Therefore, a great number of plant biofactories (i.e., places where clonal plants are produced in great scale using plant tissue culture) are dispersed around the world producing enormous amount of clonal plants. Although these plants are more expensive than the conventional plant production, some advantages encourage their use, such as production during all year (independent of seasons), selected elite genotypes, plants free of diseases and use of reduced spaces. Moreover, the plant tissue culture is an important tool that allows the production of primary and secondary metabolites in environmental controlled conditions, independent of climatic conditions [2]. However, there is a great cost to establish and to maintain the operations in these biofactories.

The development of new cheaper methods for *in vitro* culture are necessary to the biofactories to become more profitable. In this context, some advances have been done, including: (1) automated methods of *in vitro* culture, using bioreactors [3]; which reduce costs due to the use of a liquid culture medium that eliminates the use of agar, that is the most expensive component in the culture medium [4] and decreases the amount of labour (2) New culture media using industrial wastewater such as vinasse are used as source of mineral nutrients [1,5] (3) The substitution of the conventional sources of the MS culture medium nutrients [6] by locally available fertilizers [7] (4) Growth room using natural light to culture *in vitro* plants and (5) the use of macro and micronutrients as precursors for plant growth regulators (e.g., Zinc as precursor of indole-3-acetic acid); (6) alternative methods to produce plant growth regulators, such as jasmonates, gibberellic acid and indole-3-acetic acid [8, 9, 10] and (7) chemical sterilization methods of the culture medium [11] has contributed to decreased costs of production.

Gibberellic acid (GA_3) is a plant hormone that belongs to the group of gibberellins [12]. Plants produce low amounts of GA_3. Therefore, commercially, this hormone is produced by microorganisms (i.e., by fermentation) or by chemical synthesis. Nowadays, it is industrially produced by submerged fermentation, but this process results in low yield with high production costs. One alternative to reduce the costs of GA_3 production is the solid-state fermentation (SSF) that allows the use of agro-industrial residues [9]. SSF was carried out using *Fusarium moniliforme* and citric pulp (CP) extract giving 5.9 g $GA_3.kg^{-1}$ dry CP after three days of fermentation [13]. Nevertheless, the purification process of this molecule elevates the production costs, consequently studies comparing the purified GA_3 with GA_3 fermented extract not purified must be carried out to evaluate the possibility of use these fermented extracts in the more different ways.

The aim of this research was to evaluate the effects of GA_3 fermented extract obtained by SSF and comparing its effects against the synthetic GA_3 (analytical degree) for *in vitro* elongation and rooting of shoots of two ornamental bromeliads: *Nidularium innocentii* and *Nidularium procerum*. The fermented extract stability at room temperature was also studied to analyze its shelf life.

MATERIAL AND METHODS

Gibberellic acid sources

The synthetic GA_3 was a commercial powder (analytical degree ≥ 90%) obtained from Sigma-Aldrich Chemical (USA) and was dissolved with 1N NaOH and solubilized with distilled water. The GA_3 fermented extract was obtained via SSF by *Fusarium moniliforme* strain LPB 03 using citric pulp supplemented with sucrose as substrate.

The production was carried out in column bioreactors (250 mL volume) with forced aeration (30mL.min^{-1}) with 70% humidity during 5 days. GA_3 was extracted with sodium phosphate buffer (pH 7.4) and filtered. This material was precipitated with 20% ethanol and the supernatant containing GA_3 was not purified [13]. Quantitative determination of GA_3 of the crude fermented extract was performed by spectrophotometry at 254 nm absorbance [14].

***In vitro* establishment of bromeliads**

The seed disinfection of *Nidularium procerum* and *Nidularium innocentii* was performed as proposed by [12]. This process consisted of the seed immersion in 70% ethanol (v/v) during one minute, followed by immersion in commercial bleach (1% active chlorine) for 20 min, and rinsed three times with distilled sterilized water. The germination medium was MS [6], with 30 g.L^{-1} sucrose and solidified with 6 g.L^{-1} agar. Seedlings were multiplicated on MS medium supplemented with 30 g.L^{-1} sucrose, 2 μM naphthalene acetic acid (NAA), 4 μM 6-benzylaminopurine (BAP) and solidified with 6 g.L^{-1} agar.

***In vitro* elongation and rooting using different GA_3 sources and levels**

Shoots (2 cm height) from clusters (multiple shoots) propagated *in vitro* were used as explants. Basal medium was composed by MS with 30 g.L^{-1} sucrose and solidified with 7 g.L^{-1} agar. This experiment was organized in a two-way ANOVA (2 × 3) in which the factor A consisted of two sources of GA_3 (analytical degree and fermented extract) and factor B consisted of GA_3 levels which were 0, 4.1 and 8.2 μM. The root number, rooting percentage, height of the aerial part (cm), leaf number, lateral shoot number, fresh weight (g) and lateral shoot percentage were evaluated after 50 days of *in vitro* culture. It was used 1.5 mL.L^{-1} and 3.0 mL.L^{-1} of the GA_3 fermented extract that corresponds to the levels 4.1 and 8.2 μM GA_3, respectively. All GA_3 solutions were sterilized by microfiltration (0.22 μm) and added to cooled autoclaved media at about 60ºC inside the laminar flowhood.

Stability of the GA_3 present in fermented extract at room temperature

A sample of GA_3 fermented extract was collected after the fermentation and placed in 5 mL tubes. These tubes were sealed with thread caps and stored at room temperature in the dark. These tubes were used to quantify weekly the GA_3 (present in fermented extract) until 260 days. The GA_3 content was determined according to methodology described by [14]. The data were expressed in percentage relative to the GA_3 initial amount, which was considered as 100%.

Culture conditions and statistical analysis

The pH of all media was adjusted to 5.8 and then were autoclaved at 1.1 kg.cm^{-2} and 121ºC for 20 min. The cultures were kept at 25 ± 2ºC under white fluorescent light (28 μmol m^{-2} s^{-1}) with a 16 h photoperiod. The experimental design was completely randomized in a factorial arrangement (two-way) with five replicates of five explants. The data was submitted in a normality analysis for the Lilliefors´s test, and submitted to the analysis of variance (ANOVA) followed by Duncan's test at a $P<0.05$. All statistical analyses were done following the procedures of the software SOC [16]. Variables from counting were transformed to $\sqrt{x+0.5}$ and variables from percentage were transformed to $\arcsin\sqrt{x/100}$.

RESULTS AND DISCUSSION

Effects on *N. innocentii*

The best result for the height of the aerial part was obtained in the presence of GA_3, nevertheless, the levels of 4.1 and 8.2 did not differ statistically. However, there were no statistical differences between the type of sources of GA_3 (Table 1). Similar results were observed for the same species, but cultivated in double-phase medium using GA_3 (analytical degree), wherein the best result for this species was 7.3 cm of height obtained at 8.2 µM [15]; due to the culture in the double-phase medium promotes a higher growth of the plants. This difference can be due to the higher ability of absorption. This larger absorption can be attributed to the lack of physical barriers in the culture medium, which increases the explant contact with nutrients, plant growth regulators and water whereby it usually increases the *in vitro* growth [17].

Table 1. Characteristics of *Nidularium innocentii* cultivated on media supplemented with different sources of GA_3 (analytical degree and fermented extract) and levels of GA_3 after 50 days of *in vitro* culture.

	Root number			**Rooting percentage**		
GA_3	**Gibberellic acid source**			**Gibberellic acid source**		
(µM)	**GA_3 (AD)[1]**	**GA_3 (FE)**	**Mean**	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**
0	5.4 abA[2]	5.2 aA	5.3	100 aA	100 aA	100
4.1	5.0 bA	2.7 bB	3.8	96 aA	72 bB	84
8.2	5.7 aA	1.4cB	3.5	100 aA	41.6 cB	70.8
Mean	5.4	3.1		98.6	71.2	
	Height of the aerial part (cm)			**Leaf number**		
GA_3	**Gibberellic acid source**			**Gibberellic acid source**		
(µM)	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**
0	4.4	5.1	4.7 b	16.9 aA	16.5 aA	16.7
4.1	5.1	5.7	5.4 a	10.7 bB	14.3 bA	12.5
8.2	5.6	5.7	5.6 a	11.0 bB	13.3 cA	12.1
Mean	5.0 A	5.5 A		12.8	14.5	
	Number of lateral shoots per explant			**Fresh weight (g)**		
GA_3	**Gibberellic acid source**			**Gibberellic acid source**		
(µM)	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**
0	2.0 aA	2.1 aA	2.0	0.476	0.354	0.415
4.1	0.7 bA	0.9 bA	0.8	0.318	0.437	0.377
8.2	0.6 bA	0.5 cA	0.5	0.340	0.304	0.322
Mean	1.1	1.1		0.378	0.365	

	Lateral shoots (%)		
GA_3	**Gibberellic acid source**		
(µM)	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**
0	88 aA	88 aA	88
4.1	53 bA	40 bA	46.5
8.2	48 bA	61.2 bA	54.6
Mean	63	63	

[1] Source of gibberellic acid: AD = Analytical degree GA_3 and FE = Fermented extract containing GA_3.
[2] Treatments followed by same lower case letters in the same column and same capital letters in the line do not differ significantly ($P<0.05$) by Duncan´s test.

Major rooting percentage of tissues was verified in the absence of GA_3 (0 µM), however there were no statistical differences among the levels using the analytical degree GA_3 source, nevertheless higher levels of GA_3 present in the fermented extract

decreased the rooting percentage (Table 1). The best results for the root number were obtained in the absence of GA_3 (0 µM) and 8.2 µM GA_3 (analytical degree). The presence of GA_3 (crude fermented extract) in the media decreased the root number (Table 1). Similar result was found in *Lavandula angustifolia* cultivated on GA_3 fermented extract; however, this other GA_3 fermented extract was partially purified; this lower rooting was attributed to the presence of impurities that remained in the fermented extract even after the partial purification [9]. Different species had presented different behavior related to root number when they were cultured with different levels of GA_3. In potato (*Solanum tuberosum*), a concentration of 0.248 $mg.L^{-1}$ GA_3 had doubled the root number per plantlet (5.6 roots per plant) when compared to control (2.3 roots per plant) [18]. But bromeliads, normally root in culture medium free of plant growth regulators, such as in *Dyckia macedoi* [19], *D. agudensis* [20], *D. maritima* [21], *Vriesea scalaris* [22] and *Orthophytum mucugense* [23]. The main use of GA_3 in bromeliads is to promote the shoot elongation to easy the manipulation due to little size of explants in multiple shoots (clusters).

The concentration of the fermented extract containing GA_3 seems also to have an impact over the inhibition of root induction as observed in the reduction of rooting percentage and root number in *N. innocentii*, it was used 1.5 $mL.L^{-1}$ and 3.0 $mL.L^{-1}$ of GA_3 fermented extract that corresponds to 4.1 and 8.2 µM GA_3, respectively. The increase in the levels of unknown compounds present in this fermented extract has an inhibitory effect on the rooting, as also was demonstrated in *L. angustifolia*, which high concentrations inhibited completely the rooting (0%), at level of 31.2 $mL.L^{-1}$ that corresponded to 1.0 $mg.L^{-1}$ GA_3 [9]. However, the GA_3 level (analytical degree) did not influence the rooting percentage (Table 1).

The GA_3 presence on the culture medium, independent of the GA_3 source and level used; decreased the percentage of lateral shoots and number of lateral shoots.The highest value for percentage of lateral shoots and number of lateral shoots per explant was observed in a culture medium free of gibberellic acid (0 µM GA_3) (Table 1). However, when the aim is to promote the elongation and rooting, normally the lateral shoots are undesirable. Thus, the lowest values for percentage of lateral shoots were observed in a culture medium supplemented with 4.1 µM GA_3 (fermented extract) and 8.2 µM GA_3 (analytical degree) (Table 1). The lowest values for number of lateral shoots were observed in a culture medium supplemented with 8.2 µM GA_3 (fermented extract and analytical degree). The lateral shoots produced in this phase of elongation and rooting is a result from prior multiplication phase due to cytokinins presence [21], whereas cytokinins are primarily responsible for breaking apical dominance and consequent lateral shoot induction [24].

The GA_3 levels of both sources (analytical degree and fermented extract) decreased the leaf number (Table 1). Different results for leaf number were found in potato (*Solanum tuberosum*) where the concentration of 0.248 $mg.L^{-1}$ of GA_3 increased the leaf number (7.3) as compared to control (3.3) [18]. These results found in bromeliads possibly are associated with a larger energetic spend used for elongation of the aerial part of the plant, which results in a decrease of roots, leaves and shoots. The best result for leaf number was obtained at 0 µM GA_3 (analytical degree and fermented extract) (Table 1). The leaf number per explant is a characteristic normally correlated to shoot number per explant and a great number of leaf number per explant can be important when leaf explants were used to induce new shoots or calli, as some micropropagation or somatic embryogenesis protocols were established, as is the case of *Cryptanthus* sp. [25], *Neoregelia cruenta* [26], *Ananas comosus* [27] and *Vriesea scalaris* [22].

The GA_3 level and sources (analytical degree and crude fermented extract) did not influence the fresh weight (Table 1). Different results were obtained for *Lavandula angustifolia* cultivated *in vitro* with different GA_3 sources (analytical degree or

purified partially fermented extract). In this case, there was a decrease in the fresh weight proportionally to the increase of the GA_3 level. Nevertheless, there was no difference between the GA_3 sources [9].

Effects on *N. procerum*

The best result for the height of the aerial part was obtained in the presence of GA_3, nevertheless, the levels of 4.1 and 8.2 did not differ statistically; however, there were statistical differences between the type of source of GA_3, being the GA_3 analytical degree superior than GA_3 fermented extract (Table 2). *N. procerum* seems to be more sensitive than *N. innocentii* to certain compounds present in fermented extract, what influences on the height of the aerial part. Nevertheless, according to this result it is possible that different species possess different answers to GA_3 crude fermented extract, presenting results completely or partially suitable.

Table 2. Characteristics of *Nidularium procerum* cultivated on media supplemented with different sources of GA_3 (analytical degree and fermented extract) and levels of GA_3 after 50 days of *in vitro* culture.

	Root number			**Rooting percentage**		
GA_3	**Gibberellic acid source**			**Gibberellic acid source**		
(µM)	**GA_3 (AD)[1]**	**GA_3 (FE)**	**Mean**	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**
0	3.8 aA[2]	3.8 aA	3.8	92	96	94
4.1	2.4 bA	2.3 bA	2.3	95	96	95.5
8.2	2.4 bA	2.2 bA	2.3	88	88	88
Mean	2.8	2.7		91.6 A	93.3 A	
	Height of the aerial part (cm)			**Leaf number**		
GA_3	**Gibberellic acid source**			**Gibberellic acid source**		
(µM)	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**
0	4.9	4.8	4.8 b	16.1	16.9	16.5
4.1	7.7	6.3	7.0 a	10.5	14.9	12.7
8.2	7.8	6.2	7.0 a	11.6	15.9	13.7
Mean	6.8 A	5.7 B		12.7 B	15.9 A	
	Number of lateral shoots per explant			**Fresh weight (g)**		
GA_3	**Gibberellic acid source**			**Gibberellic acid source**		
(µM)	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**
0	1.8	2.2	2.0	0.227	0.207	0.217 a
4.1	0.8	2.4	1.6	0.189	0.189	0.189 a
8.2	0.8	2.6	1.7	0.212	0.197	0.204 a
Mean	1.1 B	2.4 A		0.209 A	0.197 B	

	Lateral shoots (%)		
GA_3	**Gibberellic acid source**		
(µM)	**GA_3 (AD)**	**GA_3 (FE)**	**Mean**
0	68	65.5	66.7
4.1	44	77	60.5
8.2	56	65.5	60.7
Mean	56 B	69.3 A	

[1] Source of gibberellic acid: AD = Analytical degree GA_3 and FE = Fermented extract containing GA_3.
[2] Treatments followed by same lower case letters in the same column and same capital letters in the line do not differ significantly ($P<0.05$) by Duncan´s test.

The best result for the root number was obtained in the absence of GA_3 (0 µM). The presence of GA_3 in the medium decreased the root number. GA_3 sources did not influence the root number in *N. procerum* (Table 2). The rooting of *N. procerum* was not inhibited by impurities present in the fermented extract.

The percentage of lateral shoots varied from 88 to 96% (Table 2). The GA_3 sources presented statistical differences, being the fermented extract superior to analytical degree. The highest value for the leaf number per explant was observed in a culture medium free of gibberellic acid (16.9 leaves per explant). However, there was no statistical difference in GA_3 levels, but there was statistical difference in GA_3 sources, being the fermented extract superior to analytical degree (Table 2).
The fresh weight did not present significant difference for GA_3 levels (Table 2). Similar result was observed in nodular cultures of *Vriesea reitzii* cultivated on MS medium supplemented with 5 or 10 µM GA_3 [28]. However, the GA_3 sources presented statistical difference, being the analytical degree superior to fermented extract (Table 2). Micropropagation has been employed to enhance the mass production, quality, and sterilized condition of the targeted plant [29]. Nevertheless, a great yield in biomass is desired when the aim of the work is to produce primary or secondary metabolites; moreover, micropropagated plants with larger biomass can promote higher survival rates during the acclimatization.
The percentage of lateral shoots and number of lateral shoots per explant presented statistical difference in the GA_3 sources, being the fermented extract superior than analytical degree (Table 2). This result can be interesting in the case which the multiplication is desired. Nevertheless, it is known that cytokinins are the the most indicated plant growth regulators for promotion of shoot multiplication as demonstrated in innumerous studies performed in bromeliads, such as *Vriesea scalaris* [22], *Nidularium procerum* and *Nidularium innocentii* [15], *Aechmea blanchetiana* and *Aechmea distichantha* [30] and *Neoregelia concentrica* [31]. Moreover, the use of the GA_3 present in the fermented extract to induce multiplication could be a good alternative to cytocynins case the relation cost/benefit be advantageous, considering wheter there is cost reduction.

Stability of the GA_3 present in fermented extract at room temperature
Stability tests were performed at room temperature with GA_3 fermented extract to test the possibility to produce and commercialize a liquid extract. GA_3 concentration remained constant till the 14th day of storage. After this time, there was a slight decline in the amount of GA_3 reaching 92% that was stable during 260 days of storage. The GA_3 stability of the fermented extract produced and tested in the present study is considered satisfactory, with a loss of activity of less than 10% after 260 days at room temperature. Furthermore any stabilizers or additives could be added to the extract, which could further enhance the storage life of the product.

CONCLUSIONS

The GA_3 present in the fermented extract possess the same effect of purified GA_3 (analytical degree) for the increase of the height of aerial part of shoots of *N. innocentii*, but not for the *N. procerum*. The GA_3 fermented extract influences negatively the rooting in *N. innocentii*, while GA_3 analytical degree practically does not interfere. On the other hand, in *N. procerum*, both the GA_3 sources reduce the root number and do not interfere in rooting percentage. GA_3 crude fermented extract is an alternative to reduce costs replacing the GA_3 analytical degree (i.e., most expensive), however, its results can vary depending of the species and parameter evaluated. The fermented extract storage was at room temperature during 260 days and its shelf life presented a suitable stability, maintained 92% GA_3 initial amount.

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