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Determination of Optimal Condition to Obtain the Bromelain from Pineapple Plants Produced by Micropropagation

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ABSTRACT

This study aimed to obtain the condition of maximum bromalein activity in different parts of pineapple plants produced in vitro, *by micropropagation. The sStems and leaves of* Pérola *and* Imperial *cultivar plants were evaluated after three and eight months of* in vitro *cultivation in Murashige and Skoog medium without growth phytoregulator, macerated in potassium phosphate buffer at different pH values (5.7, 6.7 and 7.7). Total protein and proteolytic activity were determined in the samples after three- and eight-month cultivation periods. For both the cultivars, the best results were obtained at pH 5.7 in extraction media.* Pérola *cultivar, showed higher bromelain activity in the leaves cultivated* in vitro *for three months (0.0194U/mL) while in the* Imperial *cultivar, it was higher in the stem after eight months (0.0179 U/mL).* Imperial *cultivar showed higher bromelain activity than the* Pérola's*.*

Key words: Enzymatic activity, bromelain, Pérola pineapple, Imperial pineapple, production *in vitro*

INTRODUCTION

Bromelain is a protease of vegetal origin obtained from several species of the *Bromeliaceae* family. *Ananas comosus L Merril* is the main source of bromelain, a product of great commercial value that is found in its fruits, stem, leaves and roots. It is used in food and pharmaceutical industries. Its efficacy has been reported to heal several health problems such as angina, indigestion and respiratory problems (Ferreira et al. 2011; Hale et al. 2005). Commercially bromelain is obtained from the pineapple stem. The fruit is also a good option for its source as it has higher proteolytic activity than in the stem. A good quantity of fruit residue is generated by the pine apple processing factories, which can be used as raw material (Ferreira et al. 2011; Fileti et al. 2009 and 2010; Lopes et al. 2012; Parks 2001).

In vitro cultivation is an important procedure in the multiplication of different species, This technique can be applied on the industrial scale. The micropropagation, besides offering a greater amount of seedlings in a short period of time, enables total control of environmental conditions during the propagation and preservation of matrix

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plants without risk of infection (Pasqual et al. 1998, 2001; Moreira et. al. 2003; Barboza et al. 2006). This technique can be an option for large - scale production of vegetal material from pineapple plant aiming to commercially exploit the bromelain enzyme.

Considering the increasing development of biotechnology, it has become necessary to develop new techniques to separate and purify biomolecules, such as enzymes and proteins of animal, vegetal and microbial origin. These techniques should maintain the chemical and physical properties of molecules and, further, should be highly efficient in the purification. Thus, the study on both purification and alternative extraction methods is of great interest, aiming to obtain more economically feasible processes. Hence, solid-liquid extraction applied to a technological process is extremely important for the development of new protein extraction and purification methods (Ferreira et al. 2011; Fileti et al. 2009 and 2010; Lopes et al. 2009; Silveira et al. 2009). This study aimed to quantify bromelain activity in different parts of pineapple plants produced *in vitro*.

MATERIALS AND METHODS

Micropropagation

Pérola and Imperial cultivars were provided by the EMBRAPA Tabuleiros Costeiros (Aracaju, SE, Brazil). The micropropagation was carried out using MS medium (Murashige and Skoog 1962) without growth phytoregulator as described by Corrêa et al. (2009), Quoirin et al. (2008), Radmann et al. (2011) and Scherwinski-Pereira et al. (2009). The MS medium was supplied with 30 g/L of sucrose and 7 g/L of agar-agar. The pH was adjusted at 5.8 and the medium was autoclaved at 120°C for 15 min. Inoculum was under s were done on aseptic conditions and light intensity of 52 µmoles/m^2.s at 27 ± 2ºC for 16 h. The combination of growth regulators for adaptive phase were 2.5 µmol and 0.625 µmol concentrations of amino benzyl purine (BAP) and acetil-naftalenic acid (ANA), respectively. These in the multiplication phase were 10 and 25 µmol of concentrations of BAP and ANA, respectively. During the multiplication phase, the plants were grown for three and eight months (Corrêa et al. 2009; Quoirin et al. 2008; Radmann et al. 2011; Scherwinski-Pereira et al. 2009).

Obtaining the Enzyme

The raw extract was obtained from the leaf and stem of pineapples *in vitro* using 1g mass which was macerated in 5 mL of 0.2M phosphate buffer at different pH (5.7, 6.7 and 7.7) and filtered twice in gauze (Almeida et al., 2002; Ferreira et al. 2011; Fileti et al. 2009 and 2010; Lopes 2005; Lopes et al. 2009 and 2012). Total protein was determined according to Bradford's method (Bradford 1976; Ferreira et al. 2007; Padilha et al. 2012; Severo Júnior et al. 2007) and enzymatic activity by the casein digestion method, or Kunitz's method, with modifications (Ferreira et al. 2011; Lopes 2005; Lopes et al. 2009; Silveira et al. 2009). The step to obtain the enzyme is shown in Figure 1.

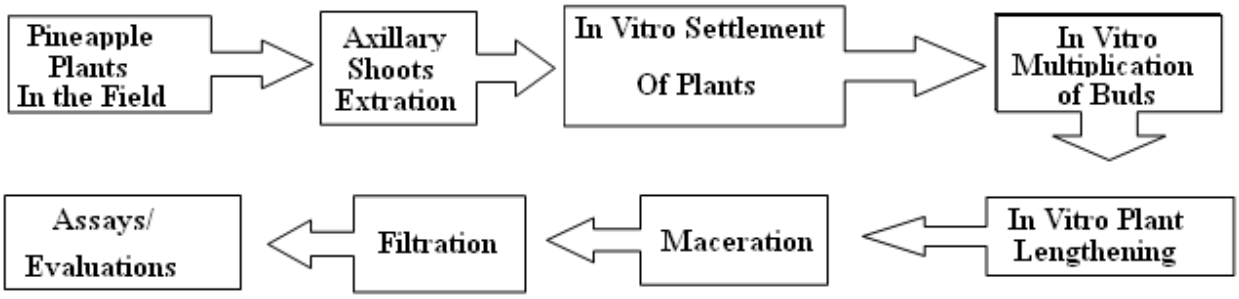

Figure 1 – Flowchart for *in vitro* production of plants and obtainment of bromelain enzyme raw extract.

Preparation of Buffer Solutions

Monobasic potassium phosphate (24.36, 12.72 and 2.2g) and dibasic potassium phosphate (1.18, 6.2 and 10.68g) were dissolved in 1000 mL of distilled water, respectively for pH 5.7, 6.7 and 7.7. Its pH was adjusted with phosphoric acid or potassium

hydroxide (Morita and Assumpção 1995; Ferreira et al. 2011; Ferreira et al. 2007; Severo Júnior et al. 2007; Padilha et al. 2012; Severo Jr et al. 2009).

Preparation of the Samples

The leaves and stems from the *in vitro Pérola* and *Imperial* cultivars were macerated separately at different pH values (5.7, 6.7 and 7.7) of phosphate buffer filtered twice in gauze and evaluated (Kiss et al. 1995; Moreira et al. 1999 and 2006; Pasqual et al. 1998 and 2001).

RESULTS AND DISCUSSIONS

Table 1 presents the results of bromelain activities. In *Pérola* cultivar, the activity was lower when a buffer solution with higher pH was used and there was a reduction in the enzymatic activity when the plant cultivation time was longer. There was also a reduction in total protein as the cultivation time was longer, except at pH 7.7

When comparing the cultivation time in a medium with no growth regulator, the concentration profiles were similar for both the ages and only differed in the basic region, where the leaf's extract whose cultivation time was longer showed an almost constant behavior as the three-month old leaves. pH 5.7 showed the best results for all the cultivar parts and any cultivation period of time, demonstrating to be the optimal pH for this enzyme. Considering the best cultivations conditions for the *Pérola* cultivar, consisting of pH 5.7 and a 3-month cultivation for the leaves, enzymatic activity was 0.0194 U/mL and total protein had a concentration of 10.342 mg/L.

Table 2 shows the results for Imperial cultivar. There was a reduction in bromelain enzyme activity when the pH in the extraction solution was increased; indicating that pH 5.7 also should be optimal for this cultivar. However, enzymatic activity increased as the cultivation time increased either in the leaves or in plant stems, an inverse behavior of that presented by *Pérola* cultivar. Total protein showed a reduction when the cultivation time was longer, which was similar to *Pérola*'s cultivar.

Table 1 - Bromelain's activity and protein concentration in the leaves and stems of *Pérola* cultivar regarding distinct cultivation times and pH values for buffer solution.

pH	Activity (U/mL)				Proteins (mg/L)			
	Leaves		Stem		Leaves		Stem	
	3 months	8 months	3 months	8 months	3 months	8 months	3 months	8 months
5.7	0.0194	0.0050	0.0053	0.0097	10.342	10.679	6.3268	4.4458
6.7	0.0073	0.0008	0.0098	0.0040	12.796	11.652	7.5910	5.6837
7.7	0.0012	0.0002	0.0015	0.0010	1.5061	11.497	0.2559	5.4563

Table 2 - Bromelain's enzymatic activity and protein concentration in leaves and stems of Imperial cultivar considering different cultivations times and phosphate buffer pH values.

pH	Activity (U/mL)				Proteins (mg/L)			
	Leaves		Stem		Leaves		Stem	
	3 months	8 months	3 months	8 months	3 months	8 months	3 months	8 months
5.7	0.0107	0.0112	0.0148	0.0180	8.759	8.759	4.402	4.087
6.7	0.0083	0.0167	0.0000	0.0076	12.530	8.146	6.270	4.048
7.7	0.0030	0.0114	0.0067	0.0149	12.136	11.248	6.0381	3.995

It was also noticed that the enzymatic activity was higher in the stems than in the leaves and total protein was higher in the latter. A good enzymatic activity for low protein concentrations is considered ideal as a high specific activity is obtained (activity/protein mass). So, the optimal condition to obtain bromelain enzyme from Imperial cultivar in an *in vitro* production was the one in which the enzyme was extracted from the stem cultivated for eight months in a buffer phosphate at pH 5.7, whose enzymatic activity was 0.0148 U/mL and total protein concentration was 4.087 mg/L.

Results of the Figure 2 corroborated with the analysis of Tables 1 and Table 2. It showed that high activities were found in the leaves for the *Pérola* pineapple variety and steam sample for the *Imperial* pineapple variety and both were high values for three months cultivation. When comparing the two varieties, the *Imperial* cultivar showed higher production potential for bromelain production than the *Pérola* cultivar.

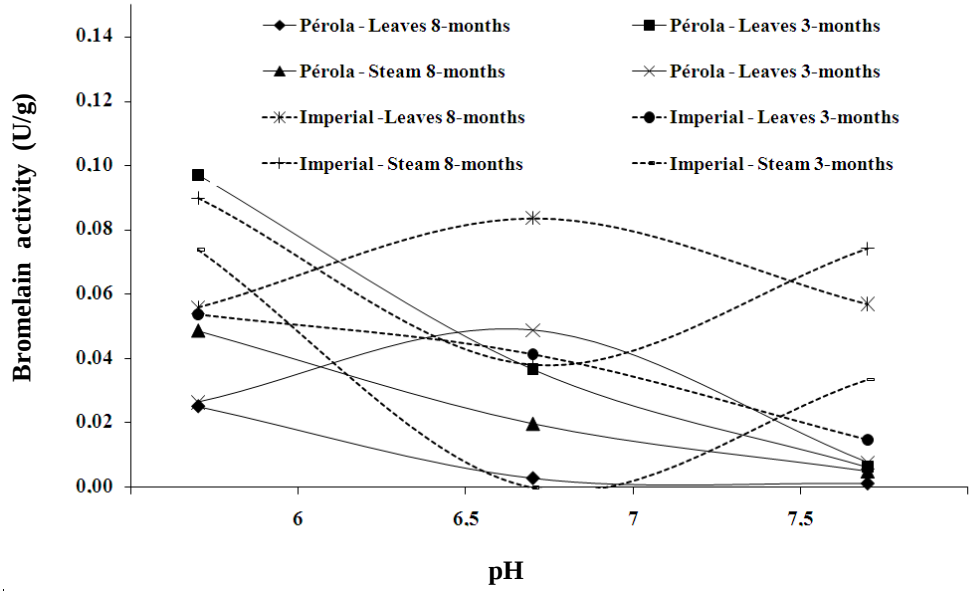

Figure 2 - Enzymatic activities for unity of pineapple plants.

Alcantara et al. (2011) and Pereira et al. (2011) found that longer period affected negatively the *in vitro* explant aspect, leading to a decrease in the survival of the plant during acclimatization for *Eucalyptus grandis*, *Eucalyptus urophylla* and apple plants. However, Radmann et al. (2011) observed that between 20-40 days of growth of 'Flordaguard' rootstock, longer cultivaion time was best. Scherwinski-Pereira et al. (2009) showed that effect of the growth time varied the seasons for the potato plants. The crop period was fast in spring-summer (96 days) than autumm-winter (107 days). The micropropagation is used to maximize the plant growth. This technique decreases the production time and increases the productivity and resistance of plants to the diseases, helps in the regeneration, control in propagation genetic improvement, etc. There are other techniques such as genetic transformation that can be incorporated in a genetic breeding program in order to increase yields, shorten production cycles and promote the development of high quality plantations (Alcantara et al. 2011; Corrêa et al. 2009; Pereira et al. 2011; Radmann et al. 2011; Scherwinski-Pereira et al. 2009).

CONCLUSIONS

Studies aimed to quantify bromelain obtained from the leaves and stems extracts of *in vitro Perola* and Imperial pineapple cultivars using a simple extraction with phosphate buffer at pH 5.7, 6.7 and 7.7, enabled the following conclusions:

- The proteolytic enzymatic activity in the pineapple plants *in vitro* extracts varied in function of extraction medium pH, variety, part and age of the plant.
- The enzymatic activity increased as the pH of the phosphate buffer pH decreased.
- There was significant variation in protein concentration amount between the pineapple parts evaluated.
- For both the cultivars, the best pH for the extraction was 5.7.

- The best cultivation time for *Perola* cultivar was the three month and for Imperial cultivar, it was eight months.
- The best part to obtain bromelain in *Perola* cultivar was its leaf, while in *Imperial* cultivar, it was its stem.
- *Imperial* cultivar had higher bromelain production potential than the *Perola* cultivar.
- The method used in this study was simple and economical, and efficient to evaluate the activity in the enzymatic preparations as well.

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