Adv. Hort. Sci., 2020 34(2): 233-240 DOI: 10.13128/ahsc-7848

*Short note*

# Extraction of total protein from shoots of *Cereus* morphological variants (Cactaceae) for proteomic analysis

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*Key words*: cactus, phenotypic variants, protein extraction, SDS-PAGE, succulent tissues.

**Abstract: Since there is a hypothesis that qualitative and/or quantitative differences of specific proteins may be associated with morphological variants in cacti of the *Cereus* genus (phenotypes erect, *tortuosus* and *monstruosus*), in current study we tested three different methods for protein extraction from shoots of the phenotypic variants to obtain protein fractions for further proteomic analysis. The TCA/acetone method for protein extraction revealed a larger number of well-defined bands in SDS-PAGE system than the methods with phenol. The quantification of protein extracted by TCA/acetone ranged between 0.488 (*tortuosus*) and 2.92 µg·mL$^{-1}$ (*monstruosus*). Although the use of phenol is the most appropriate procedure for protein extraction from recalcitrant tissues, results have shown that extraction buffer containing two antioxidant agents (EDTA and β-mercaptoethanol) and PMSF to prevent protein degradation was efficient to avoid proteolysis and lower protein yield, than using TCA/acetone precipitation for protein extraction from shoots of *Cereus* sp. The use of extraction buffer with appropriate combinations of antioxidant agents, phenol-complexing agents, and protease inhibitors may be an efficient alternative for proteins extraction from succulent and recalcitrant tissues (such as cactus plants) using a simple protein extraction method.**

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**Citation:**
FERNANDES V.N.A., MANGOLIN C.A., NEVES A.F., SOUSA NOGUEIRA F.C., ZENI NETO H., MACHADO M.F. P.S., 2020 - *Extraction of total protein from shoots of Cereus morphological variants (Cactaceae) for proteomic analysis.* - Adv. Hort. Sci., 34(2): 233-240.



**Data Availability Statement:**
All relevant data are within the paper and its Supporting Information files.

**Competing Interests:**
The authors declare no competing interests.

Received for publication 4 September 2019
Accepted for publication 16 January 2020

## 1. Introduction

Since the 1960s the study on proteins from plant tissues is frequently restricted by extraction procedures from different tissues (Loomis, 1969; 1974; Kelley and Adams, 1977). Different procedures for protein extraction have been reported for different tissues and plant species to obtain

protein fractions which are adequate for electrophoresis and polypeptides characterization on gel. Taxa and tissues have their own set of endogenous tannins, phenols and phenoloxidases which interfere differently with protein stability. Protein extraction from cactus tissues is particularly an arduous process due to the abundance of complex heteropolysaccharides, coupled to endogenous tannins, phenols and phenoloxidases in the shoot tissues of adult plants. Heteropolysaccharides confer high viscosity to the extraction solution after shoot-tissue homogenization and hinder the solubilization of proteins.

Protein extraction has been a primordial stage for the studies on proteomics. The detection of proteins and protein variants found in cells of a given tissue under specific conditions (functional genomic) (Wilkins *et al*., 1996; Westermeier and Naven, 2002) requires adequate solubilization and stability of protein fractions. The first step should establish a procedure for protein extraction from shoots, or rather, for the proteome analysis in shoots of phenotypic variants of cactus from genus *Cereus*. The *Cereus* plants have been mainly used as forage for ruminants (Silva *et al*., 2011) and fruits and pulp are used in the preparation of cookies, pies, and pastries (Almeida *et al*., 2007). Several studies have revealed the importance of the *C. peruvianus* species as fruit crop (Mizrahi, 2014). While the *C. jamacaru* plants constitute a wild natural resource in the semiarid region of Northeastern Brazil, an industrial and economic importance has been attributed to the *C. peruvianus* species cultivated in the Southern region of Brazil (Alvarez *et al*., 1995; Barros and Nozaki, 2002). Medicinal importance is also attributed to *C. peruvianus* since arabinogalactan extracted from the gum has been indicated for the treatment of gastric ulcers (Tanaka *et al*., 2010). Phenotypic variants of cacti from the genus *Cereus*, tagged *tortuosus* and *monstruosus* varieties, are frequently cultivated together with plants featuring typical columnar-erect shoots in home gardens and public parks and squares in Brazil´s southern region. Although the origin of the *tortuosus* and *monstruosus* varieties has not been reported in the literature, the qualitative and/or quantitative differences of specific proteins may be associated with the morphological variants.

Since the success of a proteomic experiment depend on the correct identification of proteins, three different methods described by He and Wang (2008) for protein extraction from shoots of the three phenotypic variants of *Cereus* were employed in the current study. The methods described by He and Wang (2008) were used in protein extraction from *Aloe vera* tissues, a succulent and recalcitrant plant similar to cacti. It is expected that protocols described by these authors may be suitable for protein extraction and quantification from shoots of *Cereus* plants.

## 2. Materials and Methods

Samples of *Cereus* plants with typically erect shoots and plants of the varieties *tortuosus* and *monstruosus* (Fig. 1), cultivated in south Brazil (in Maringá PR Brazil, at 23°25'38" S; 51°56'15" W), were collected from home gardens and maintained in the Experimental Botanic Garden of the State University of Maringá (altitude 554.9 m; 23°25'S; 51°25'W). Pieces of shoots (2 g) from erect, *tortuosus* and *monstruosus* plants (four plants of each morphology) were collected and used as samples for protein extraction. The cuticle and the fractions of cells with chlorophyll were removed from the pieces of shoot to minimize the contamination of the samples by polysaccharides.

Fresh shoot sections (2 g) of each *Cereus* plant with erect shoots (E1-E4) and plants of the varieties *tortuosus* (T1-T4) and *monstruosus* (M1-M4) were ground to a fine powder in liquid nitrogen and homogenized in 2 mL buffer 0.02 M Tris-HCl pH 7.5, 0.25 M sucrose, 0.01M ethylene glycol tetraacetic acid (EDTA), 0.001 M phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100 and 2% ß-mercaptoethanol, following method by He and Wang (2008). Proteins were extracted from two samples of each E1-E4, T1-

Fig. 1 - Different morphologies of *Cereus peruvianus* plants showing stems: erect (A), *tortuosus* (B), *monstruosus* (C).

T4, and M1-M4 plants (Table 1).

After homogenization, 1.0-mL aliquots from each *Cereus* plants were transferred to 2 mL microtubes and prepared by three procedures: i) TCA-acetone precipitation; ii) phenol extraction, and iii) improved phenol extraction method according to described by He and Wang (2008).

Protein was quantified by the fluorometric method in a Fluorometer Qubit® 1.0 using Qubit® Protein Assay kit from Life Technologies. Polyacrylamide gel (12%) was prepared with 16.2 mL of 30% acrylamide and 0.8% bis-acrylamide dissolved in 4 mL of 1.5 M Tris-HCl, pH 8.8, 107 µL 10% SDS, 5.7 mL twice-distilled water, 320 µL 2% ammonium persulfate and 16 µL TEMED. Stack gel was prepared at a final concentration of 5%, pH 6.8: 3 mL of 10% acrylamide and 5% bis-acrylamide dissolved in 3 mL of 0.24 M Tris-HCl pH 6.8, 30 µL twice-distilled water, 250 µL 2% ammonium persulfate and 3 µL TEMED. Further, 25 mM Tris/200 mM glycine, pH 8.3, and 0.1% SDS were employed in the electrode chambers.

Samples were taken from the freezer and 2 µL loading dye [20% glycerol 10 mM, Tris-HCl 1.5 M pH 8.8, 10% ($w \cdot v^{-1}$) bromophenol blue, 10% SDS ($w \cdot v^{-1}$), β-mercaptoethanol 2% ($v \cdot v^{-1}$), and twice-distilled water q.s.p.] were added to 8 µL of sample and applied to the gel. Electrophoresis was performed in Tris-glycine buffer at 200 volts, for thirty minutes, for the stacking gel, followed by two hours and thirty minutes for the separation of the proteins. After electrophoresis, the gel was fixed in a fixation solution (40% methanol; 70% acetic acid) and stained in silver 20% following protocol by Laemmli (1970). After migration, gels were stained, photographed and the images were analyzed with GelAnalyzer 19.1 software (http://www.gelanalyzer.com/) to transform electrophoretic bands into peaks, to calculate Rf and estimate the molecular weight of proteins.

The protein quantification ($\mu g \cdot \mu L^{-1}$) by the fluorometric method in duplicate of morphological variants of *Cereus* with shoots erect (E1-E4), *tortuosus* (T1-T4), and *monstruosus* (M1-M4) obtained by extraction with TCA/acetone method described by He and Wang (2008) were analyzed using the software R (R Core Team, 2019) with the packages: i) "nortest" (Gross and Ligges, 2015) to verify the normality of the errors by the Lilliefors test (Lilliefors, 1969), ii) "car" (Fox and Weisberg, 2019) to verify the independence of errors by the Durbin-Watson test (Durbin and Watson, 1951), and iii) the "ExpDes" package (Ferreira *et al*., 2018) to verify the homogeneity of variances by the O´Neill and Matthews test (O´Neill and Matthews, 2000). All these procedures were admitting the error at 1% of significance.

## 3. Results and Discussion

The TCA/acetone method for protein extraction from shoots of *Cereus* plants revealed a larger number of well-defined bands in SDS-PAGE system than the methods with phenol and improved-phenol extraction. In the TCA/acetone method, each homogenized tissue (plant tissues with shoots erect, *tortuosus* and *monstruosus*) in the extraction buffer was centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was then recovered and placed in a new tube where ¼ of the volume of acetone containing 50% trichloroacetic acid (TCA) was added. The solution was incubated for 2 h, at -20°C and centrifuged again at 15,000 rpm for 40 min, at 4°C. The supernatant was then discarded and the pellet was

Table 1 - Protein quantification ($\mu g\ \mu L^{-1}$) by the fluorometric method in duplicate of morphological variants of *Cereus* with shoots erect (E1-E4), *monstruosus* (M1-M4), and *tortuosus* (T1-T4) obtained by the extraction with TCA/acetone method described by He and Wang (2008)

| Erect | | *Monstruosus* | | *Tortuosus* | |
|---|---|---|---|---|---|
| sample | $\mu g \cdot \mu L^{-1}$ | sam | $\mu g \cdot \mu L^{-1}$ | sample | $\mu g \cdot \mu L^{-1}$ |
| E1 | 0.824 | M1 | 2.64 | T1 | 1.090 |
| | 0.658 | | 2.68 | | 0.990 |
| E2 | 0.732 | M2 | 1.71 | T2 | 0.768 |
| | 0.766 | | 1.91 | | 1.150 |
| E3 | 0.964 | M3 | 2.26 | T3 | 1.740 |
| | 0.874 | | 2.40 | | 1.520 |
| E4 | 1.100 | M4 | 2.54 | T4 | 0.488 |
| | 1.480 | | 2.92 | | 0.592 |

washed three times with acetone containing 0.2% 1,4-Dithiothreitol (DTT). Pellets were then dissolved in 50 µL lysis buffer (8 M urea, 4% NP-40 and 1% DTT) and stocked at -20°C.

The quantification of protein extracted by TCA/acetone method was made in duplicate (two samples of each shoot morphology: erect, *tortuosus* and *monstruosus*) and ranged between 0.488 (*tortuosus*) and 2.92 µg·mL$^{-1}$ (*monstruosus*) (Table 1).

The normality of the errors by the Lilliefors test (Lilliefors, 1969), the independence of errors by the Durbin-Watson test (Durbin and Watson, 1951) and the homogeneity of variances by the O´Neill and Matthews test (O´Neill and Matthews, 2000) showed that there were no restrictions by *p*-values regarding analysis of variance (ANOVA) procedure (Table 2).

Significant differences (at 1% level) in protein concentrations within and between *Cereus* plants with the erect, *tortuosus* and *monstruosus* morphologies were detected by ANOVA procedure (Table 3).

The non-significant experimental error indicates that the sampling error (different morphologies) may justify the differences in protein concentrations between *Cereus* plant morphologies. Non-significant experimental error assures the researcher more reliable results because the effect of uncertainty is smaller (Patterson, 1946; Cochran and Cox, 1957; Snedecor and Cochran, 1980).

Table 2 - p-values of the normality of errors by the Lilliefors test (Lilliefors, 1969), independence of errors by the Durbin-Watson test (Durbin and Watson, 1951) and the homogeneity of variances by the O´Neill and Matthews test (O´Neill and Matthews, 2000)

| Procedure | Normality | Independence | Variance |
|---|---|---|---|
| Lilliefors | 0.8897 | | |
| Durbin-Watson | | 0.0379 | |
| O´Neill and Matthews | | | 0.4089 |

Table 3 - Mean Squares (MS) of the comparison of protein concentrations from the three *Cereus* morphologies (erect, *tortuosus* and *monstruosus* shoots) and their respective sampling and experimental mistakes

| | MS |
|---|---|
| Morphologies | 5.2477 ** |
| Sample error | 0.2969 ** |
| Experimental error | 0.0251 |

** *p*-value <0.01.

A higher protein concentration in *monstruosus* plants than in erect and *tortuosus* plants has been detected by Tukey´s test (Tukey, 1953) employed to compare the averages of protein concentrations in erect, *tortuosus* and *monstruosus* plants (Table 4). Figure 2 also illustrates the highest protein concentration in *monstruosus* plants and shows the amplitude of concentration rates within each plant.

Differences in protein concentrations in *Cereus* plants with the erect, *tortuosus* and *monstruosus* morphologies indicate that it is necessary to evaluate the protein concentration in more than one plant with the same morphology to specify the average protein concentration of each morphology. Differences in protein concentrations among *Cereus* plants with erect, *tortuosus* and *monstruosus* morphologies detected in current study support the hypothesis that qualitative and/or quantitative differences of specific proteins may be associated with morphological variants in cacti of the *Cereus* genus, and may be used to justify a proposal for further proteomic analysis.

The phenol extraction method described by He and Wang (2008) revealed a number of protein frac-

Table 4 - Mean protein concentrations (µg µL$^{-1}$) from the three *Cereus* morphologies (erect, *tortuosus* and *monstruosus*) revealed by the Tukey´s test

| Morphology | Mean protein concentraction (µg·µL-1) |
|---|---|
| *Monstruosus* | 2.3825 a |
| *Tortuosus* | 1.0423 b |
| Erect | 0.9248 b |

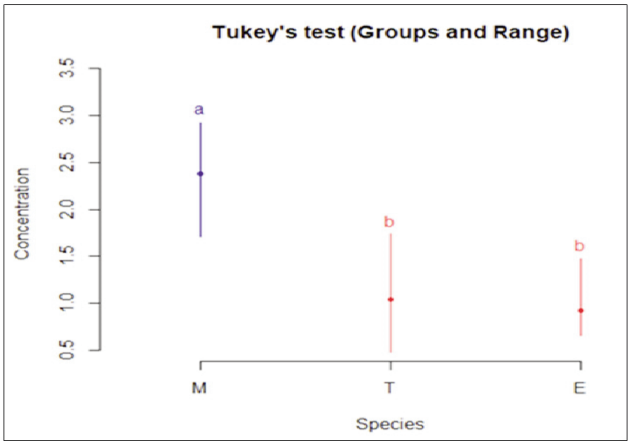


Fig. 2 - Amplitude of the concentration values observed within *monstruosus* (M), *tortuosus* (T), and erect (E) plants of *Cereus*.

tions smaller than the number of protein fractions detected by the TCA/acetone precipitation method in *Cereus* plants, while protein fractions were absent with the improved phenol extraction method described by He and Wang (2008)(Fig. 3). The GelAnalyzer software was useful to create calibration curves and to estimate the quantity of the protein from a band. Figure 4 shows the calibration curve that make a correspondence between the quantity of the protein loaded on each lane and the areas of the peaks of each lane (the software name the area of the peak as raw volume and the conventional units are in pixels). The correlation coefficients of such calibration curves were higher than 0.99 for proteins with molecular masses ranging from 24 until 180 kDa (Table 5). However, data obtained by MS (Mass Spectrometry analysis) indicated proteins with molecular weights varying from 220 to 15 kDa. In gel regions without clear band distinction in 1DE SDS-PAGE were detected considerable amounts of protein identified by MS. Preliminary Mass Spectrometry analysis identified 753 proteins extracted by the TCA/acetone precipitation method in the *Cereus* plants (unpublished results).

Improved phenol extraction method was described as the most appropriate procedure for pro-

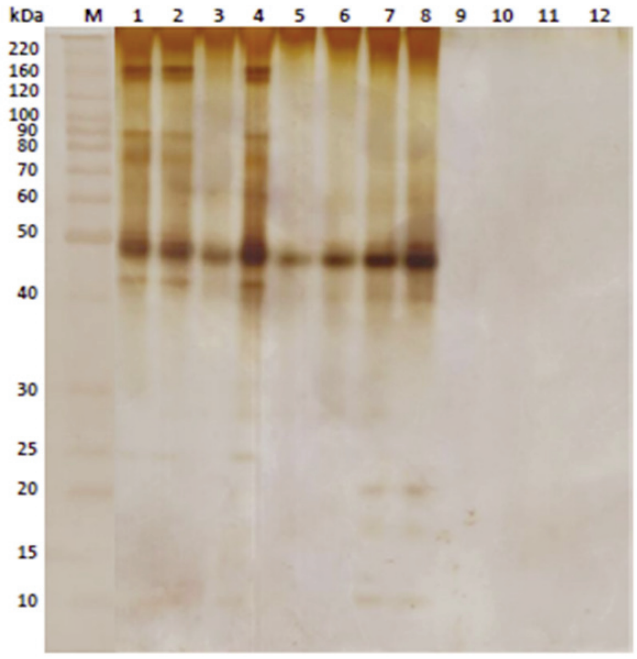


Fig. 3 - Protein extraction from shoots of *Cereus* with erect (samples 1-2, 5-6, 9-10), *tortuosus* (samples 3, 7, 11) and *monstruosus* (samples 4, 8, 12) morphologies, with TCA/acetone method (samples 1-4), with phenol extraction method (samples 5-8) and with the improved-phenol extraction method by He and Wang (2008) (samples 9-12), in the SDS-PAGE system 12%.

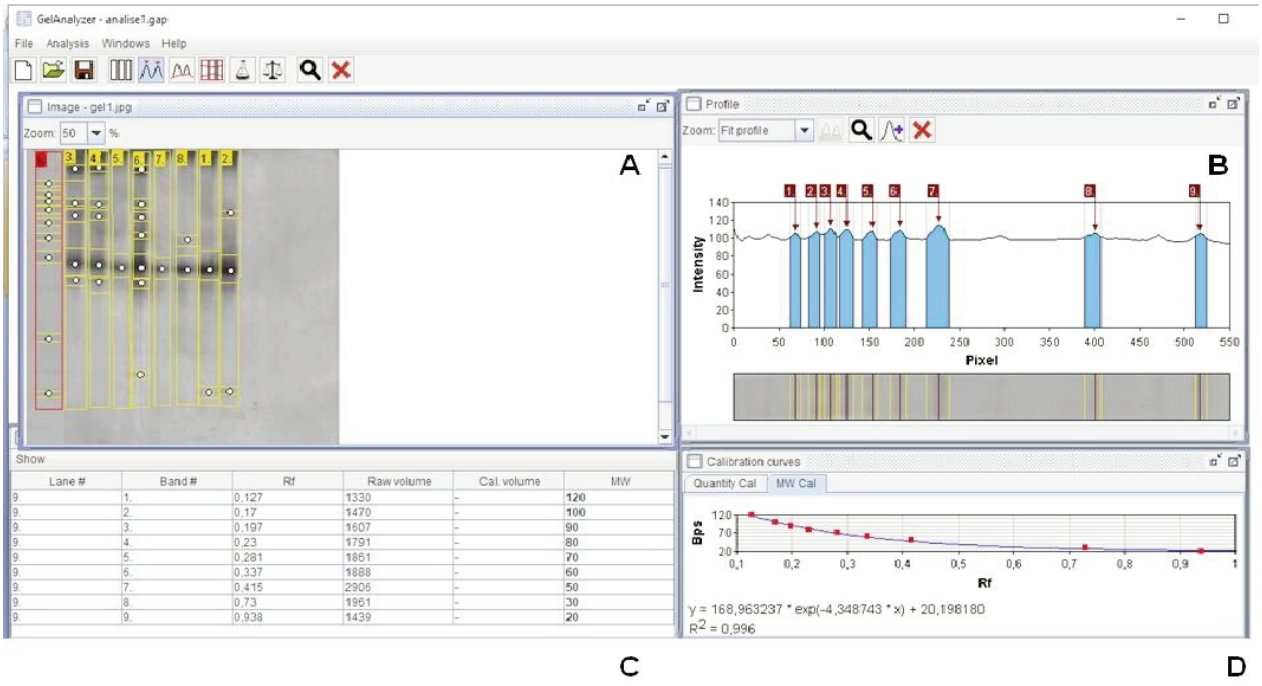


Fig. 4 - The main panels of Gel Analyser software showing the image of the entire electrophoresis gel (panel A); the bands from the selected lane from panel A are transformed in peaks (panel B) and their area are computed in panel C, as raw volume (conventional units). Based on the molecular weights of the markers (in panel D), the correspondence between the molecular mass and the migration (as Rf) of the protein is presented.

Table 5 - Quantity of the protein loaded on each lane detected with TCA/acetone method (samples 1-4) and with phenol extraction method (samples 5-8) in the SDS-PAGE system 12% from the *Cereus* plants

| Lane | MW of each band (KDa) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Lane 1 | - | - | - | - | - | - | 45 | - | 24 |
| Lane 2 | - | - | - | 77 | - | - | 45 | - | 24 |
| Lane 3 | - | 145 | 90 | 77 | - | - | 45 | 39 | - |
| Lane 4 | 180 | 145 | 90 | 77 | - | - | 45 | 39 | - |
| Lane 5 | - | - | - | - | - | - | 45 | - | - |
| Lane 6 | - | 145 | 90 | 77 | 61 | - | 45 | 39 | 24 |
| Lane7 | - | - | - | - | - | - | 45 | - | - |
| Lane 8 | - | - | - | - | - | 58 | 45 | - | - |

tein extraction from *Aloe vera* tissues, a succulent plant, similar to cacti. Phenol has also been considered as the most effective for the removal of unwanted interfering substances from the protein samples of tissues from other recalcitrant plants (He and Wang, 2008; Pavoković *et al.*, 2012; Riffel *et al*., 2012). It has been postulated that, in the case of particularly recalcitrant tissues, acetone and TCA/acetone precipitation do not sufficiently remove nucleic acids, carbohydrates and polyphenols, which cause co-precipitation and degradation of proteins. The phenol method, although more laborious and time-consuming, resulted in higher protein yield and with the lowest contamination rate of samples than the TCA/acetone precipitation method alone for protein extraction from recalcitrant tissues (He and Wang, 2008; Pavoković *et al.*, 2012; Wu *et al.*, 2014).

However, results from the current study are indicative that the use of an extraction solution containing two antioxidant agents (EDTA and β-mercaptoethanol) and PMSF to prevent protein degradation was efficient to avoid proteolysis and lower yield of proteins using TCA/acetone precipitation for protein extraction from shoots of *Cereus* sp. Although the use of phenol is reported to be the most appropriate procedure for protein extraction from recalcitrant tissues, a significant proportion on cellular proteins may be lost from the extracts during the extraction procedure with phenol. The phenol extraction method involves more steps than the TCA/acetone method while a 'minimum-steps´ method is preferred with protein extraction procedures (Isaacson *et al*., 2006). The TCA/acetone method described by He and Wang (2008) involved only one precipitation, two centrifugations and three washes after homogenization of the tissues, whereas nine steps (including one overnight precipitation at -20°C) were reported for phenol method. Thus, the use of the TCA/acetone method described by He and Wang (2008) represented a minimum-steps method and, thus, a shorter time for the extraction of proteins from shoot tissues of *Cereus* plants.

A relatively simple extraction buffer containing one phenol-complexing agent (PVP) and two antioxidant agents (β-mercaptoethanol and EDTA) was also used for electrophoresis of the several isozymes in shoot tissues of the cactus *Cereus peruvianus* (Mangolin and Machado, 1997), while phenol and ammonium acetate were employed for the protein extraction from callus tissues of *C. peruvianus* (Mangolin *et al.*, 1999) for two-dimensional electrophoresis of callus tissues grown in culture media containing different concentrations of auxin and cytokinin. Only one phenol-complexing agent (PVP) and a cocktail of protease inhibitors (Roche, USA) were used for protein extraction from tissues of the cactus *Mammillaria gracilis* grown in vitro (Rogic´ *et al*., 2015). The TCA/acetone/β-mercaptoethanol method plus protease inhibitors (Roche, USA) was also employed for protein extraction from several species of succulent plants and some cactus species (*Lophocereus marginatus*, *Mammillaria magnimamma* and *Opuntia ficus-indica*) instead of phenol precipitation method (Lledías *et al*., 2017). The evidence from these studies actually demonstrates that, in the case of tissues with different levels of interfering substances, different phenol complexing agents and different antioxidant agents are required. The physiological state of each tissue is the decisive factor of a greater or lesser complexity in protein extraction processes. As types and concentrations of polysaccharides, polyphenols and other secondary metabolites may be induced in response to environmental factors, different types and concentrations of phenol-complexing agents and antioxidant agents may be needed for the same tissue in different environmental conditions. According to Wendel and Weeden (1989), extraction solutions for tissues with moderate

levels of interfering substances require at least two phenol-complexing and two antioxidant agents, while at least four phenol-complexing and three antioxidant agents are needed for tissues with high levels of interfering substances.

## 4. Conclusions

The simplest method (TCA/acetone precipitation), described by He and Wang (2008), and the use of two antioxidant agents and a protease inhibitor showed a number of protein fractions greater than the number of protein fractions detected with the phenol method in shoots of *Cereus* analyzed in current study (plants with erect shoots and plants of the varieties *tortuosus* and *monstruosus*). The authors´ expectation is to use this simple method of extracting proteins for proteomic analysis of the phenotypic variants from the genus *Cereus*. However, the use of phenol or other phenol-complexing and antioxidant agents to extract proteins from shoots of other *Cereus* plants grown in different regions or in different environmental conditions (different seasons of the year, e.g.) may be needed. Consequently, the proposal to establish a 'universal protocol' for succulent plants, such as cacti, seems unattainable. Therefore, testing with extraction buffers and precipitation methods, which may be time-consuming and laborious, seems indispensable for further analysis of other *Cereus* plants in a proteomic-based approach.

## Acknowledgements

The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasília, DF, Brazil) for financial support (CAPES; Finance Code 001) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Grant No. 306508/2013-3.

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