academicJournals

Vol. 13(34), pp. 3531-3537, 20 August, 2014
DOI: 10.5897/AJB2014.13953
Article Number: A38E1E846845
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Two-dimensional profiling of *Xanthomonas campestris* pv. *viticola* proteins extracted by four different methods

Myrzânia de Lira Guerra[1]*, Carolina Barbosa Malafaia[2], Túlio Diego da Silva[2], Rosa de Lima Ramos Mariano[1], Márcia Vanusa da Silva[2] and Elineide Barbosa de Souza[3]

[1]Departamento de Agronomia/Fitossanidade, Universidade Federal Rural de Pernambuco - UFRPE, 52171-270, Recife, PE, Brazil.
[2]Departamento de Bioquímica, Biologia Molecular, Universidade Federal de Pernambuco, Av. Professor Moraes Rego, 1235, Cidade Universitária, Recife, 50740-560, Brazil.
[3]Departamento de Biologia/Microbiologia - UFRPE, 52171-030, Recife, PE, Brazil.

Received 30 May, 2014; Accepted 4 August, 2014

An efficient method for protein extraction is a prerequisite for the successful implementation of proteomics, which is being used as a tool in the study of the interaction between plants and phytopathogens. With the objective to optimize a method of protein extraction for proteomic analysis of the phytobacterium *Xanthomonas campestris* pv. *viticola*, the efficiency of four methodologies were compared, based on the two-dimensional gel electrophoresis profile (2D-PAGE). Trizol®, phenol, centrifugation and lysis methods were tested and through quantitative and qualitative analysis, the most suitable method to obtain high-quality protein was selected. All methodologies enabled the extraction of a significant amount of proteins; nevertheless, the centrifugation method allowed obtaining the highest concentration of solubilized proteins. However, the analysis of the 2D-PAGE gel images revealed a larger number of spots in the lysis method when compared to the others. Taking into consideration the quality of the results and the practical advantages of the lysis method, this is recommended as the best option for total protein extraction of *X. campestris* pv. *viticola* for proteomic studies.

Key words: Bacterial canker, *Vitis vinifera*, proteomics, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis (2D-PAGE).

INTRODUCTION

Xanthomonas campestris pv. *viticola* (*Xcv*) (Nayudu) Dye is the causal agent of bacterial canker, one of the most important grapevine (*Vitis vinifera* L.) diseases, responsible for severe damage and representing a serious potential risk to Brazilian viticulture (Silva et al., 2012). Outside of Brazil, this disease occurs only in India (Jambenal et al., 2011) and Thailand (Buensanteai, 2004), where, anyway, severe losses have not yet been

*Corresponding author. E-mail: myrzgue@gmail.com. Tel: +55 81 9973-3785. Fax +55 81 3320-6205.

recorded. The characteristic symptoms of the disease are cankers in the branches, petioles and stems. Small dark and angular lesions are observed in the leaves; these lesions necrose large areas of the leaf when they coalesce. The veins become necrosed, particularly on the lower surface of the leaf blade (Nayudu, 1972; Trindade et al., 2007). Vein necrosis is an important symptom for diagnosis of the disease when the leaf lesions are atypical and cankers are absent. The berries of infected plants are non-uniform in size and color and may exhibit necrotic lesions (Rodrigues et al., 2011). For the successful control of bacterial canker of grapevine, it is necessary to understand the characteristics of *X. campestris* pv. *viticola* and the pathogenesis mechanisms involved in this plant-pathogen interaction, which have not yet been fully clarified (Tostes et al., 2014). Thus, proteomics technologies can integrate the basic knowledge necessary for the understanding of the mechanisms that phytobacteria use to cause diseases in their host (Norbeck et al., 2006).

Proteomics is defined as the analysis of proteins expressed by a cell or any biological sample at a given time and under specific conditions (Dierick et al., 2002). Proteins are functional molecules that play key roles in cells (Görg et al., 1995), being important for comprehensive understanding of any biological system (Beranova-Giorgianni, 2003). In comparison with genomic studies, investigations of the proteome provide detailed information, such as the abundance of proteins and post-translational modifications (Galdos-Riveros et al., 2010).

Among the various technologies used for the investigation of protein expression on a large scale, two-dimensional gel electrophoresis (2D-PAGE) stands out. This method separates proteins using a relative isoelectric point and molecular weight on its mobile base in a polyacrylamide gel matrix (Kim et al., 2007). The spots generated are used to create databases. However, it is necessary to obtain high quality protein samples, that is, free from contaminants (high levels of salts, nucleic acids, polysaccharides, phenolic compounds, pigments and other compounds) that can interfere with 2D-PAGE (Chan et al., 2002, 2004a, 2004b). Thus, an efficient method of extraction is a prerequisite for the successful implementation of proteomics (Mehmeti et al., 2011) for studies of the plant-pathogen interaction and continues to be a challenge for scientists (Natarajan et al., 2005). In this context, four methodologies to extract proteins from the phytobacterium *X. campestris* pv. *viticola* were tested, in order to optimize the sample preparation for two-dimensional electrophoresis.

MATERIALS AND METHODS

Culture conditions

The isolate of *X. campestris* pv. *viticola* (*Xcv* 137) used in the experiments was obtained from the Culture Collection of the Phytobacteriology Laboratory of the Federal Rural University of Pernambuco (Universidade Federal Rural de Pernambuco), Brazil. It was grown in 20 ml of NYD liquid medium (10 g/l dextrose, 5 g/l peptone, 5 g/l yeast extract and 3 g/l meat extract) for 24 h at 28°C under shaking (150 rpm) to obtain the pre-inoculate. The concentrations of the bacterial suspensions were adjusted to A570 = 0.4 (10^8 CFU/ml) using a spectrophotometer (Analyser 500 M, São Paulo, Brazil). Following this, 180 ml of the same NYD medium were added and the culture maintained under the same growth conditions for 24 h.

Protein extraction

In this study, four different extraction methods (Trizol®, phenol, centrifugation and lysis) were used to extract protein from a suspension of bacterial cells grown in NYD medium. The bacterial suspensions were then centrifuged at 10 000 x *g* for 5 min (CENTRIFUGE MCD-2000, Shanghai, China) and washed three times with saline solution (0.9% NaCl). The pellets were stored at 20°C and used in each method. Three biological replicates (independent cultures) were performed for each method.

Trizol method

The protocol was carried out according to manufacturer's instructions of Trizol® (Invitrogen®, Carlsbad, USA), modifying only the protein resolubilization step by using 0.5 ml of rehydration buffer without the bromophenol blue (7 M urea, 2 M thiourea, 4% CHAPS) instead of washing solution (0.3 M guanidine hypochlorite in 95% ethanol).

Phenol method

The bacterial pellet was washed in phosphate buffer (1.24 g/l K_2HPO_4, 0.39 g/l KH_2PO_4, 8.8 g/l NaCl, pH 7.2) and 0.75 ml of extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, 30 mM HCl, 50 mM EDTA, 0.1 M KCl and 40 mM DTT) was added, followed by incubation for 15 min at 28°C. The same volume of phenol was added, and after 15 min of agitation in a vortex, the suspension was centrifuged at 14 000 × *g* for 6 min at 4°C and the phenolic phase was recovered. This procedure was repeated two more times. Proteins were precipitated with the addition of five volumes of 0.1 M ammonium acetate in methanol (Mehta and Rosato, 2003). The precipitate was washed with 1 ml of 80% acetone and resolubilized as described in the previous paragraph.

Centrifugation method

Resuspension of the bacterial pellet was performed in 500 µl of extraction buffer (0.3% SDS, 200 mM DTT, 28 mM Tris-HCl and 22 mM Tris). Subsequently, the Eppendorf tube containing the cell suspension was gently agitated for 10 min at 4°C. Afterward, the sample was centrifuged at 14 000 × *g* for 10 min at 4°C, incubated at 100°C for 5 min and then cooled on ice. Next, 24 µl of assay buffer (24 mM Tris, 476 mM Tris-HCl, 50 mM $MgCl_2$, 1 mg/ml DNAse I and 0.25 mg/ml RNAse A) were added, and the sample incubated on ice for 15 min. The reaction was stopped by the addition of four volumes of ice cold acetone and precipitation of proteins was left to occur on ice for 20 min. Cell debris were removed by centrifugation at 14 000 × g for 10 min at 4°C (Giard et al., 2001). The pellet was dissolved by using 0.5 ml of rehydration buffer (7 M urea; 2 M thiourea; 4% CHAPS), and incubated at 50°C for 2 h.

Table 1. Quantification of proteins of *Xanthomonas campestris* pv. *viticola* obtained by four different methods of extraction.

Method	Concentration (µg/µl)
Centrifugation	9.1a ± 0.17
Trizol®	8.6b ± 0.17
Lysis	7.8c ± 0.17
Phenol	7.2d ± 0.15

Values are means ± standard deviation (SD) of three technical replicates. Low case letters a, b, c, d indicate significant differences using Tukey's test ($p < 0.05$).

Lysis method

Bacterial pellet was resuspended in 0.5 ml rehydration buffer (7 M urea; 2 M thiourea; 4% CHAPS), homogenized in a vortex for 5 min and centrifuged at 10 000 x *g* at 4°C for 30 min. The supernatant was then transferred to a new 1.5 ml tube (Jangpromma et al., 2007).

Quantification of proteins

The concentration of total cellular proteins obtained with each extraction method was determined by the 2-D Quant Kit, according to the manufacturer's instructions (GE Healthcare®, Piscataway, NJ, USA). Bovine serum albumin (BSA) was used as standard and the assay was performed by measuring the absorbance at 480 nm. This kit was selected as it does not interfere or interact with any chemicals used during the extractions and is therefore compatible with isoelectric focusing (IEF). The samples and the standards were read in triplicate.

One-dimensional gels (SDS-PAGE)

For the preparation of the SDS-PAGE gel the methodology of Laemmli (Laemmli, 1970) was used, which involved a 15% polyacrylamide separation gel and a 4% concentration standard molecular weight marker (High-Range Amersham™ Rainbow™) from GE Healthcare®. In each well, 30 µg of protein were loaded. Electrophoresis ran at 40 mA for 15 min and then at 100 mA for 2 h, in a vertical Owl P10DS cube (Thermo Scientific®, Hudson, New Hampshire, USA). The gels were stained using the reagent Coomassie brilliant blue (Coomassie Brilliant Blue G250) (Candiano et al., 2004) and bleached in a solution of 7.5% methanol and 5% glacial acetic acid until complete visualization of bands.

Two-dimensional gel (2D-PAGE)

The two-dimensional electrophoresis was performed in two stages according to the 2-D electrophoresis instructions of GE Healthcare®. In the first step, isoelectric focusing (IEF) was done, in which proteins were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS (w/v), 2 mM DTT, 1% IPG buffer (w/v) and 0.2% bromophenol blue).

The IEF was conducted using Ettan IPGphor 3 (GE Healthcare®) in 7 cm strips of immobilized pH gradient (IPG) ranging from 3 to 10 (Amersham Bioscience AB, Uppsala, Sweden) which were loaded with 150 µg of protein. Subsequently, the strips were balanced in reducing solutions of disulfide bridges containing DTT (dithiothreitol) and iodoacetamide (Görg et al., 1995). In the second step, 2D-PAGE electrophoresis was performed using a 15% polyacrylamide gel in an initial run of 15 mA for 20 min per gel, increasing to 45 mA per gel for about 3 h. The gels were stained as in the SDS-PAGE until complete visualization of spots.

Image analysis of gels

After staining, the gels were scanned using Image Scanner software (Amersham Biosciences) in transparency mode with a resolution of 300 dpi (dots per inch). The images of 2D-PAGE gels were analyzed using Image Master 2D-Platinum software, version 7.0 (Amersham Biosciences). The program provided the number of protein spots from each of the gels which was validated by visual inspection. For each biological replicate three technical replicates were made to confirm the reproducibility of the results.

The efficiency of the methodologies used in this study was evaluated by the qualitative parameters (resolution and intensity of bands) for SDS-PAGE and for both quantitative (amount of proteins and number of spots) and qualitative (resolution and intensity of spots, and reproducibility) parameters for 2D-PAGE.

Statistical analysis

Statistical analysis was made using the Statistix® software (version 9.0, Analytical Software, Tallahassee, USA). Data were analyzed by one way analysis of variance (ANOVA) followed by Tukey's test. In all statistical analyses, $p < 0.05$ was taken as the level of significance.

RESULTS AND DISCUSSION

For both SDS-PAGE and 2D-PAGE, which are techniques commonly used in proteomics, thorough and careful sample preparation is very important for the quantification and high resolution of proteins. Due to the different physical and chemical properties of proteins, an appropriate and standardized bioassay of a given sample, including protein extraction with different methods, favors their identification (Mehmeti et al., 2011).

In this study, four different extraction methods (Trizol®, phenol, centrifugation and lysis) were compared to determine which of them increase the solubilization of proteins of the *X. campestris* pv. *viticola*. All methodologies tested proved to be efficient in detecting a large and different ($p < 0.05$) amount of proteins (Table 1). According to Shi et al. (2013), complete solubilization of samples is the best way to achieve the goal of standardizing the recovery of proteins. The highest protein yield was obtained by the centrifugation method. The potential reasons for that may be the use of SDS in the centrifugation solution and the high temperature heating of 100°C, both recognized as critical in protein extraction (Shi et al., 2006). In the SDS-PAGE gel image analysis, the protein bands were sharp, well defined and without presenting characteristics of degradation (Figure 1).

The results of the two-dimensional gels were different

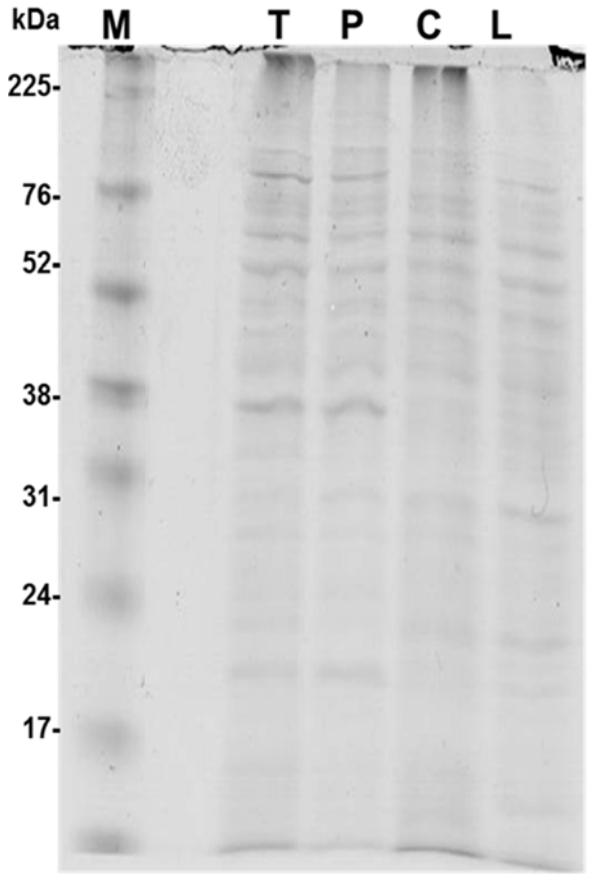

Figure 1. Representation of SDS-PAGE gel (15%) of proteins of *Xanthomonas campestris* pv. *viticola* extracted by four extraction methodologies: Trizol® (T), Phenol (P), Centrifugation (C) and Lysis (L); kDa marker (M).

in relation to the quality of the sample and numbers of spots obtained from the four methods studied (Figure 2). In the Trizol® method (Figure 2A) most of the rare and sparse spots were distributed throughout the pH range of acid and were between 76 to 24 kDa. The phenol method (Figure 2B) did not allow for a good quality sample, making focusing impossible; several horizontal stripes were observed in the gel, whose proteins were separated only by molecular weight.

According to Saravanan and Rose (2004), the presence of non-protein impurities can critically affect the quality of 2D-PAGE separation, resulting in the formation of spots and/or horizontal and vertical striations, and a notable decrease in the number of spots. In the centrifugation and lysis methods (Figure 2 C; D), the spots were more concentrated in the range of 225 to 24 kDa. The three technical replicates showed similar results for the number of spots. The lysis method presented the largest number of spots and differs significantly ($p < 0.05$) from the other extraction methods (Figure 3). Therefore, lysis method was defined as the best among the tested methods, since it allowed obtaining the highest number of spots with the best definition in 2D-PAGE gel.

Despite the lysis method did not provide the greatest concentration of proteins, as verified for centrifugation (Table 1), a high quality profile of proteins was observed in terms of resolution, number and intensity of the spots (Figure 2D). This result indicates that proteins with low abundance and high molecular weight were clearly revealed and detected in the 2D-PAGE gel by this method. Furthermore, it was observed that different sets of proteins were detected by the presence of bright spots with good resolution. The lysis method stands out not only for its greater representativeness of spots, but also for being a fast (about 1 h) and practical method, accessible to any laboratory. The most interfering materials (non-protein components) are effectively removed; the proteins are protected against degradation by proteases, thus not requiring the use of protease inhibitors. In addition, reagents used are of low cost and toxicity, when compared with other methodologies (Tan et al., 2011). This method greatly reduces the extraction time, which in turn, improves the quality of the sample.

A large amount of proteins of *X. campestris* pv. *viticola* was extracted by the phenol method (Table 1), however, the unsatisfactory results obtained from the analysis of 2D-PAGE (Figure 2B) indicated absence of different protein concentrations or inefficiency of the staining method. These two aspects are cited by Görg et al. (2004) as being more important than the concentration of protein for the success of 2D-PAGE analysis. Phenol is an effective solvent of proteins that can greatly reduce molecular interactions between proteins and other compounds that inhibit electrophoresis (Wang et al., 2007). This method has been successfully employed for protein extraction of *X. citri* subsp. *citri* (Mehta and Rosato, 2003; Soares et al., 2010), but its effectiveness was not proven under the extraction conditions of *X. campestris* pv. *viticola*.

Many techniques including physical methods and those based on detergents are available for cell disruption and protein extraction (Grabskia, 2009). These techniques can vary widely in reproducibility and in representation of the proteome and, thus, need to be adapted to the phytobacteria. Using various extraction methods, proteomic studies have been conducted with *Xanthomonas* spp. like *X. campestris* pv. *campestris* (Villeth et al., 2009), *X. oryzae* pv. *oryzicola* (Zhao et al., 2011), *X. oryzae* pv. *oryzae* (González et al., 2012), *X. axonopodis* pv. *passiflorae* (Tahara et al., 2003), *X. axonopodis* pv. *citri* (Zimaro et al., 2013) and *X. citri* subsp. *malvacearum* (Razaghi et al., 2012).

Conclusion

The results obtained in this work were satisfactory taking

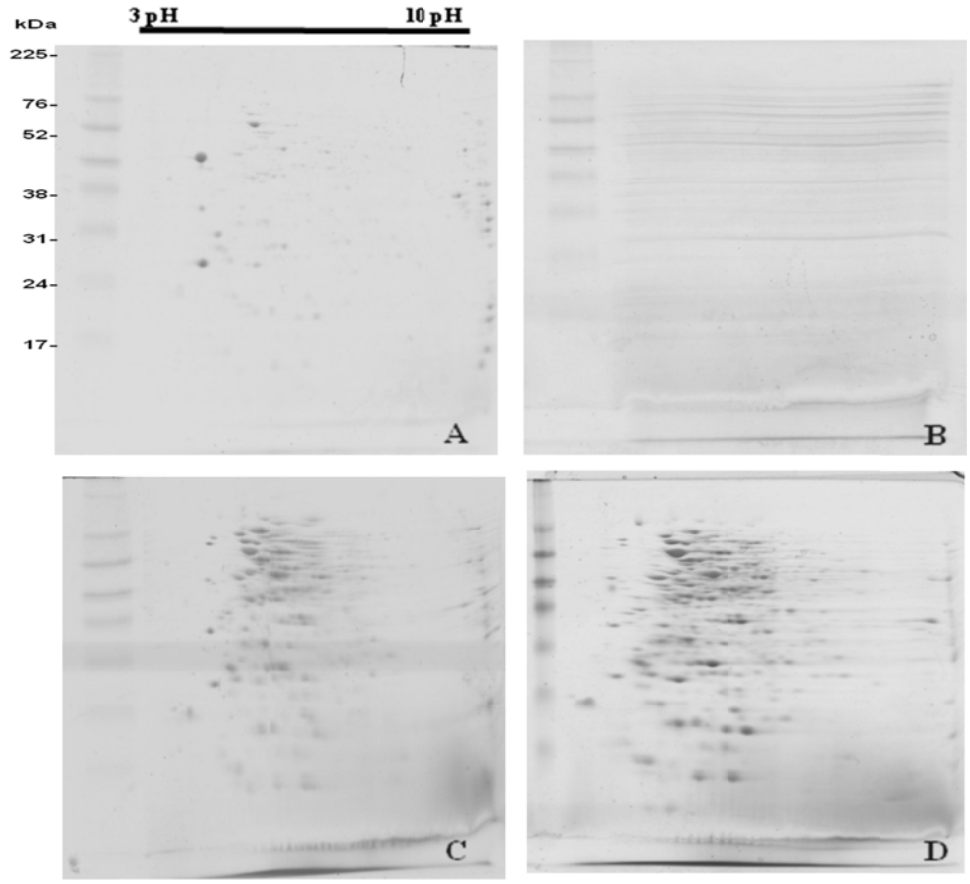

Figure 2. Representation of the 2D-PAGE gel of total proteins of *Xanthomonas campestris* pv. *viticola*, focused on strips of 7 cm pH 3-10, extracted by four methodologies: Trizol® (A), Phenol (B), Centrifugation (C) and Lysis (D).

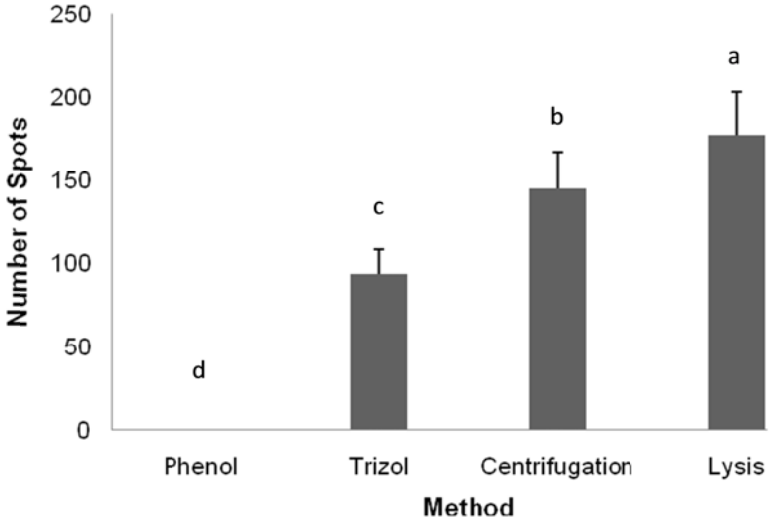

Figure 3. Number of spots identified by the Image Master 2D Platinum program in four different protein extraction methodologies of *Xanthomonas campestris* pv. *viticola*. Values are means ± standard deviation (SD) of three technical replicates. Low case letters a, b, c, d indicate significant differences using Tukey's test ($p < 0.05$).

into consideration that, in the literature consulted, no results were found of a single or a combination of methods developed for protein extraction of *X. campestris* pv. *viticola*, making this study probably the first. Therefore, considering the excellent profile of proteins obtained in 2D-PAGE analysis by the lysis method, this is recommended as the best option for total protein extraction of *X. campestris* pv. *viticola*. This extraction method can be used in proteomic research with this phytobacterium in order to study population diversity based on protein profile, detection of pathogenesis-related proteins, and biofilm formation, among others. This is an excellent opportunity to make great progresses in the understanding of plant-pathogen interaction, aiming at establishing efficient management measures of bacterial canker of grapevine.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES

Beranova-Giorgianni S (2003). Proteome analysis by two-dimensional gel electrophoresis and mass spectrometry: strengths and limitations. TRAC 22(5):273-281.

Buensanteai MN (2004). Identification, development of detection method and survey of bacterial necrosis disease of grapevine in Thailand. Ds. Dissertation, Suranaree University of Technology, Muang District.

Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG (2004). Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25(9):1327-1333.

Chan LL, Hodgkiss IJ, Lo SC (2004a). Use of two-dimensional gel electrophoresis proteome reference maps of dinoflagellates for species recognition of causative agents of harmful algal blooms. Proteomics 4(1):180-192.

Chan LL, Hodgkiss IJ, Wan JM, Lum JH, Mak AS, Sit WH, Lo SC (2004b). Proteomic study of a model causative agent of harmful algal blooms, *Prorocentrum triestinum* II: the use of differentially expressed protein profiles under different growth phases and growth conditions for bloom prediction. Proteomics 4(10):3214-3226.

Chan LL, Lo SC, Hodgkiss IJ (2002). Proteomic study of a model causative agent of harmful red tide, *Prorocentrum triestinum* I: optimization of sample preparation methodologies for analyzing with two-dimensional electrophoresis. Proteomics 2(9): 1169-1186.

Dierick JF, Dieu M, Remacle J, Raes M, Roepstorff P, Toussaint O (2002). Proteomics in experimental gerontology. Exp. Gerontol. 37(5):721-734.

Galdos-Riveros AC, Piza ART, Resende LC, Maria DA, Miglino MA (2010). Proteômica: Novas fronteiras na pesquisa clínica. Encicl. Biosf. 6(11):1-24.

Giard JC, Rince LJM, Rince A, Pichereau V, Benachour A, Leboeuf C, Flahaut S, Auffray Y, Hartke A (2001). The stress proteome of *Enterococcus faecalis*. Electrophoresis 22(14):2947-2954.

González JF, Degrassi G, Devescovi G, De Vleesschauwer D, Höfte M, Myers MP, Venturi V (2012). A proteomic study of *Xanthomonas oryzae* pv. *oryzae* in rice xylem sap. J. Proteom. 75(18):5911-5919.

Görg A, Boguth G, Obermaier C, Posch A, Weiss W (1995). Two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients in the first dimension (IPG-Dalt): the state of the art and the controversy of vertical versus horizontal systems. Electrophoresis 16(1):1079-1086.

Görg A, Weiss W, Dunn MJ (2004). Current two-dimensional electrophoresis technology for proteomics. Proteomics 4(12):3665-3685.

Grabskia AC (2009). Advances in preparation of biological extracts for protein purification. Academic Press, San Diego. pp. 285-303.

Jambenal S, Ravikumar MR, Hiremani N (2011). Evaluation of different chemicals and bioagents against bacterial leaf spot of grapevine and their effect on yield and yield parameters. Int. J. Plant Prot. 4(2):377-380.

Jangpromma N, Kitthaisong S, Daduang S, Jaisil P, Thammasirirak S (2007). 18 kDa protein accumulation in sugarcane leaves under drought stress conditions. KMITL Sci. Technol. 7(S1):44-54.

Kim IS, Yun HS, Jin IN (2007). Comparative proteomic analyses of the yeast *Saccharomyces cerevisiae* KNU5377 strain against menadione-induced oxidative stress. J. Microbiol. Biotechnol. 17(2):207-217.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Mehmeti I, Kiran F, Osmanagaoglu O (2011). Comparison of three methods for determination of protein concentration in lactic acid bacteria for proteomics studies. Afr. J. Biotechnol. 10(11): 2178-2185.

Mehta A, Rosato YB (2003). A simple method for *in vivo* expression studies of *Xanthomonas axonopodis* pv. *citri*. Curr. Microbiol. 47(5):400-404.

Natarajan S, Xu C, Carpena TJ, Garrett WM (2005). Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins. Anal Biochem. 342(2):214-220.

Nayudu MV (1972). *Pseudomonas viticola* sp. nov., incitant of a new bacterial disease of grapevine. J. Phytopathol. 73(2):183-186.

Norbeck AD, Callister SJ, Monroe ME, Jaitly N, Elias DA, Lipton MS, Smith RD (2006). Proteomic approaches to bacterial differentiation. J. Microbiol. Methods 67(3): 473-486.

Razaghi A, Hasanzadeh N, Ghasemi A (2012). Characterization of *Xanthomonas citri* subsp. *malvacearum* strains in Iran. Afr. J. Microbiol. Res. 6 (6):1165-1170.

Rodrigues Neto J, Destéfano SAL, Rodrigues LMR, Pelloso DS, Oliveira Júnior LC (2011). Grapevine bacterial canker in the state of São Paulo, Brazil: detection and eradication. Trop. Plant Pathol. 36(1):42-44.

Saravanan RS, Rose JK (2004). A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. Proteomics 4(9):2522-2532.

Shi S-R, Liu C, Balgley BM, Lee C, Taylor CR (2006). Protein extraction from formalin-fixed, paraffin-embedded tissue sections: quality evaluation by mass spectrometry. J. Histochem. Cytochem. 54(6):739-743.

Shi S-R, Taylor CR, Fowler CB, Mason JT (2013). Complete solubilization of formalin-fixed, paraffin-embedded tissue may improve proteomic studies. Proteom. Clin. Appl. 7(3-4):264-272.

Silva AMF, Menezes EF, Souza EB, Melo NF, Mariano RLM (2012). Sobrevivência de *Xanthomonas campestris* pv. *viticola* em tecido infectado de videira. Rev. Bras. Frutic. 34(3):757-765.

Soares MR, Facincani AP, Ferreira RM, Moreira LM, Oliveira JCF, Ferro JA, Ferro MIT, Meneghini R, Gozzo FC (2010). Proteome of the phytopathogen *Xanthomonas citri* subsp. *citri*: a global expression profile. Prot. Sci. 8(1):55-65.

Tahara ST, Mehta A, Rosato YB (2003). Proteins induced by *Xanthomonas axonopodis* pv. *passiflorae* in the interaction with leaf extract of the host plant (*Passiflorae edulis*). Proteomics 3(1):95-102.

Tan AA, Azman SN, Abdul-Rani NR, Kua BC, Sasidharan S, Kiew LV, Othman N, Noordin R, Chen Y (2011). Optimal protein extraction methods from diverse sample types for protein profiling by using Two-Dimensional Electrophoresis (2DE). Trop. Biomed. 28(3):620-629.

Tostes GO, Araujo JSP, Farias ARG, Frade DAR, Olivares FL (2014). Detection and cellular localization of *Xanthomonas campestris* pv. *viticola* in seeds of commercial 'Red Globe' grapes. Trop. Plant Pathol. 39(2):134-140.

Trindade LC, Marques E, Lopes DB, Ferreira MASV (2007). Development of a molecular method for detection and identification of *Xanthomonas campestris* pv. *viticola*. Summa Phytopathol. 33(1):16-23.

Villeth GR, Reis Júnior FB, Tonietto A, Huergo L, Souza EM, Pedrosa FO, Franco OL, Mehta A (2009). Comparative proteome analysis of *Xanthomonas campestris* pv.*campestris* in the interaction with the susceptible and the resistant cultivars of *Brassica oleracea*. FEMS Microbiol. Lett. 298:260-266.

Wang X, Li X, Deng X, Han H, Shi W, Li Y (2007). A protein extraction method compatible with proteomic analysis for the euhalophyte *Salicornia europaea*. Electrophoresis 28(21):3976-3987.

Zhao YC, Qian GL, Yin FQ, Fan JQ, Zhai ZW, Liu CH, Hu BS, Liu FQ (2011). Proteomic analysis of the regulatory function of DSF-dependent quorum sensing in *Xanthomonas oryzae* pv. *oryzicola*. Microbiol. Pathogenesis 50(1):48-55.

Zimaro T, Thomas L, Marondedze C, Garavaglia BS, Gehring C, Ottado J, Gottig N (2013). Insights into *Xanthomonas axonopodis* pv. *citri* biofilm through proteomics. BMC Microbiol. 13:186-200.