RESEARCH ARTICLE

STANDARDIZATION OF A STAINING METHODOLOGY FOR SUGARCANE PROTEINS TOWARDS BETTER MASS SPECTROMETRY COMPLIANCE

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Abstract

The application of proteomic tools to understand plant defense and plant-pathogen interaction is becoming inevitable. Red rot of sugarcane needs to be studied in detail in the proteomics perspective to understand the molecular basis behind systemic acquired resistance (SAR) and sugarcane defense. This study primarily focuses on identifying differentially regulated sugarcane stalk proteins in response to priming with inducer/elicitor employing two dimensional gel electrophoresis (2-DGE) and mass spectrometry (MS). Though the methodology for protein extraction from sugarcane stalks was standardized, the post-2D processes leading to MS analysis were often hindered due to the staining method currently employed. Hence, a detailed study was envisaged to explore the sensitivity of staining methods reported to be compatible with MS analysis. Among the staining methods evaluated, silver-galactose, silver-glucose and modified Kang's method were found to be the most sensitive methods. MS-compatibility of these methods needs to be evaluated rigorously before recommending it as the most suitable staining method for 2-DGE. Further, studying the regulation of proteins in inducer/elicitor primed stalks would enlighten the understanding of the molecular basis of defense and unveil SAR-mediated defense responses in sugarcane.

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Introduction

In this functional genomics era, proteomics is progressing at an unprecedented pace, complementing genomics and metabolomics. Proteomic tools are becoming inevitable in understanding pathogenicity and the key regulatory events during host-pathogen interaction. As compared to other monocots like rice, where proteomics is already an established platform and a plethora of information is available (Agrawal et al. 2005; Rakwal et al. 2009), sugarcane proteomics is yet in its infancy. However, methods for protein extraction from sugarcane stalks were established and work on sugarcane reference proteome map had been initiated in the lab (Ramesh Sundar et al. 2010). Evidence on the application of proteomic tools to study plant defense and plant-pathogen interaction is accumulating rapidly (Amey et al. 2008; Viswanathan and Ramesh Sundar, 2011). Among the several diseases of sugarcane, red rot caused by *Colletotrichum falcatum* (*Cf*) is one of the serious threats to sugarcane cultivation and sugar industry in India (Alexander and Viswanathan, 2002; Viswanathan, 2010) and it continues to be an important area to be researched in detail. Resistant varieties lack desirable agronomic traits and varieties with superior qualities often succumb to the disease. Synergistic regulation of a set of genes and eventually proteins with pivotal roles in signaling during biotic/abiotic stress could be one among the many decisive factors in determining the consequences of a host-pathogen interaction as compatible or incompatible ultimately leading to

susceptible or resistant disease reactions respectively. Studying the stringent regulation of genes/proteins during host-pathogen interaction remains to be an enthralling area of research which would not only supplement the understanding of plant defense but also concomitantly lead to better deployment of strategies for effective plant disease management.

Though modern robust 'gel free' proteomic techniques like Multidimensional Protein Identification Technology (MUDPIT), Isotope Coded Affinity (ICAT) and Isobaric Tagging for Relative and Absolute Quantitation (iTRAQ) have emerged (Kav et al. 2007; Bhadauria et al., 2010), two dimensional gel electrophoresis (2-DGE) coupled with mass spectrometry (MS) is regarded as the viable option, especially in plant systems wherein the proteome is much more complexly regulated during biotic/abiotic stresses (Que et al. 2011). MS, a technique that had initially emerged to facilitate the elucidation of elemental composition, has transformed in the recent years to study macromolecules, largely proteins/peptides (Sharon and Robinson, 2007). Regardless of the choice of proteomic technique employed to resolve proteins, protein identification by MS is the preferred tool (Fig. 1). The present study envisages the application of 2-DGE to separate proteins and the use of MS to identify differentially and consistently expressed sugarcane proteins in response to priming with inducers/elicitors, a bewildering array of compounds which triggers plant defense, i.e. systemic acquired resistance (SAR), followed by challenge inoculation with the pathogen. Extraction of proteins from the primed and inoculated cane stalks was carried out as per the standardized protocol (TCA-Acetone extraction followed by solubilization in lysis buffer) (Ramesh Sundar et al. 2010) and the proteins were stained with the conventional silver nitrate method after separation by 2-DGE. In order to evaluate the MS-compatibility of silver stained protein spots, randomly excised spots were processed for MS-analysis. From the preliminary MS-analysis data, it was inferred that the post-2D processes were often hindered due to detained digestion and extraction of peptides, probably due to detrimental cross-linkers (formaldehyde) and oxidizing agents (Shevchenko et al. 1996). Inclusion of these stringent reagents renders this method least compatible with MS analysis. Fig. 2 illustrates a schematic work flow indicating steps involved in 2-DGE and subsequent

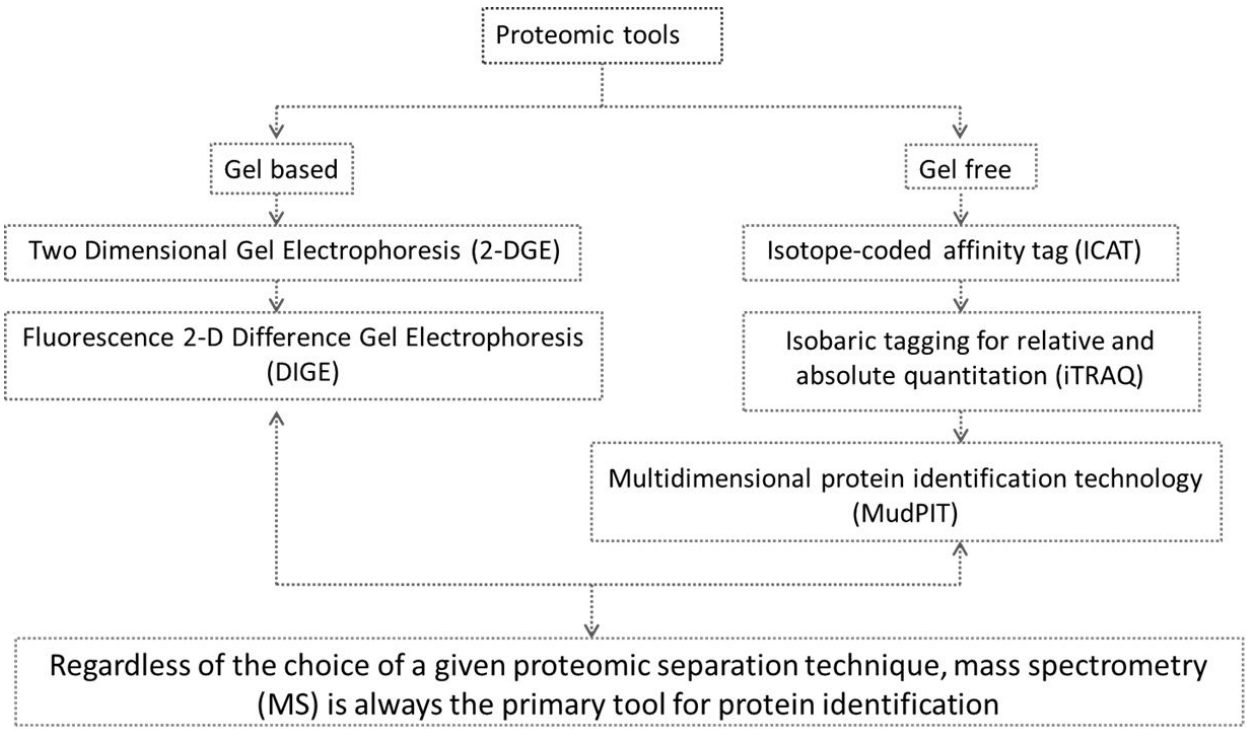

Fig. 1. An overview of the proteomic tools and the inevitability of Mass Spectrometry analysis in plant proteomic research

protein identification. Though MS is playing a significant role by complementing proteomic research in identifying proteins/peptides, successful MS-characterization of 2D resolved proteins is often hindered by the currently employed silver staining method. Stringent sample preparation, including efficient detection of resolved protein spots without altering the peptides and interfering with the subsequent MS-analysis, is a prerequisite in a 2D-based proteomic approach. Hence, a preliminary investigation was carried out to explore the sensitivity of six staining methods reported to be compatible with MS analysis, *viz.* coomassie brilliant blue (CBB), zinc-Imidazole method (Zn-Im) (Carlos Fernandez-Patron et al. 1995), modified coomassie brilliant blue (M-CBB) (Wang et al. 2007), modified Kang's method or coomassie brilliant blue G staining (MK) (Mario Pink et al. 2010), silver-glucose (Ag-glu) and silver galactose (Ag-gal) staining methods (Chevallet et al. 2008). The staining methods evaluated for sensitivity are devoid of cross linkers and stringent oxidizing/reducing agents, which are expected to result in improved compatibility with MS analyses.

Materials and methods

Priming and sampling

The elite sugarcane cultivar CoC 671, with superior agronomic traits but high susceptibility to red rot, was used in this study. Two budded setts were treated overnight with 250 µM of benzothiadiazole-S-methyl ester (BTH) and 60 µg glucose equivalents of *Cf* elicitor (Ramesh Sundar et al. 2008); mock inoculated canes (treated with water) were maintained as appropriate controls before planting. After five months of planting, the plants were foliar sprayed with respective inducers as a booster dose of priming. Inoculation of the p.PPP athogen was carried out by nodal swabbing method (Viswanathan and Samiyappan, 2002) using axenic culture of *Cf 671* pathotype isolated from CoC 671. In the nodal swabbing method, a thin layer of absorbent cotton drenched in 2ml of *C. falcatum* conidial suspension (10^6 spores/ml) was placed around the nodes immediately after removing the leaf sheaths, which ensured high humidity for the disease development. A set of sample constituted three independent biological replications. Primed stalks were collected at 72 h post-inoculation and stored at -80°C until use.

Extraction and solubilization of proteins

Following the removal of tough outer epidermis, protein extraction from the cane stalk tissue was done by the standardized protocol (Ramesh Sundar et al. 2010). Stalk tissue samples ground with liquid nitrogen yielded a fine powder; 5g of this cane stalk power was vigorously mixed with 10% trichloro acetic acid (TCA) in acetone with 20mM Dithioretiol (DTT) for 1h at -20°C with regular vortexing. The homogenate was centrifuged at 12000 rpm for 20 min. at 4°C and washed repeatedly with 0.4M DTT in acetone. Following the washing step, samples were freeze-dried and stored at -80°C until use. One hundred and fifty mg of the freeze dried powder was mixed with the appropriate volume of lysis buffer containing 7 M Urea, 2 M Thiourea, 2 % CHAPS, 0.1M DTT and IPG buffer (pH 4-7). Thoroughly mixed samples were incubated at 37°C for 1h with periodical vortexing, after which it was centrifuged at 12000 rpm for 20 min. at 4°C. The protein samples were further processed with 2D clean up kit (Amersham), after which the samples were quantified and stored at -80°C until use.

2-D Gel electrophoresis

Protein samples were passively rehydrated onto 18cm pH 4-7 linear Immobiline pH gradient (IPG) strips with appropriate volume of rehydration buffer containing 7 M urea, 2 M thiourea, 2 % CHAPS, 0.002 % bromophenol blue, 0.1 M of Dithioretiol (DTT) and IPG buffer (4-7). Isoelectric focusing (IEF) (separation of proteins based on isoelectric points) was carried out following a step-wise voltage increment procedure, i.e. 500V for 1 hour, 1000V for 1 hour and 2950 V for 21 hours until the voltage hours passed attained 45000Vhrs (GE Healthcare IPGphor II). The focused strips were subjected to a two step equilibration procedure initially with 1% DTT, followed by a second equilibration with 2.5% iodoacetamide. Second dimensional separation (based on molecular weight) of the focused strips was performed on 12% SDS-PAGE gels by a gradually increasing two step voltage program for 18h, until the dye front reached the bottom of the gel (Ettan Dalt six, GE Healthcare USA). 2D gels

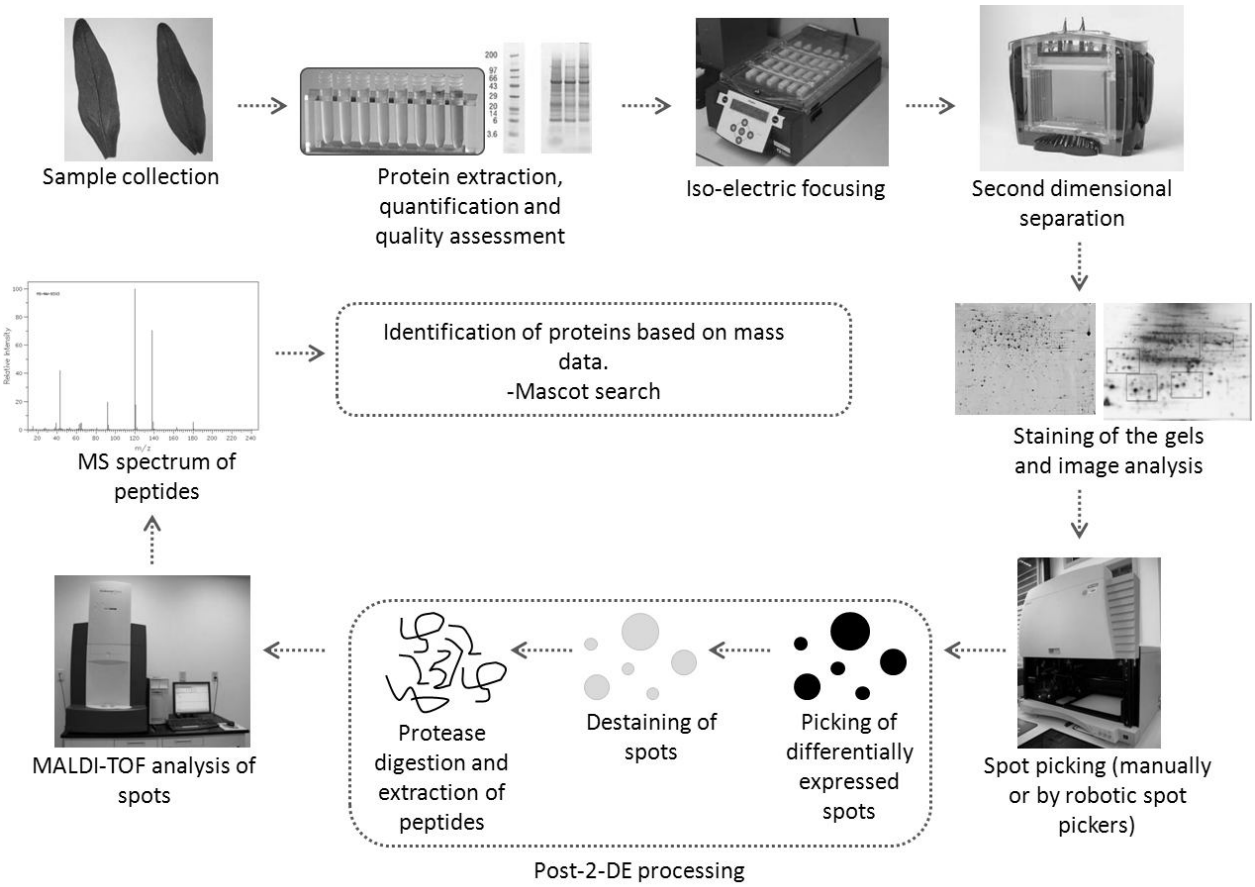

Fig. 2. 2-DGE-MS-Typical work flow

were documented using GE Healthcare Gel scanner III and the protein spots were analyzed by GE Image master 2D platinum version-7 software.

Sensitivity of the staining methods

Sensitivity of the methods was evaluated by staining bovine serum albumin (BSA) standards. For 1D SDS-PAGE, 4% stacking and 12% resolving gels were used. BSA was serially diluted and reducing concentrations (up to 10ng) resolved on SDS-PAGE gels were used to analyze the sensitivity of the methods. The staining protocols evaluated are as follows:

CBB staining

Coomassie Brilliant Blue (CBB) R, an organic disulfonated triphenylmethane dye, is the most commonly used dye to stain proteins. Though the sensitivity is limited, as CBB binds to the proteins non-specifically, reproducibility and linearity are convincing. Staining of the protein was done with 0.1% w/v CBB R 250, 50% v/v methanol and 10% v/v acetic acid and then destained with 30% methanol and 10% acetic acid.

Zinc-Imidazole method (Zn-Im)

The principle behind Zinc-Imidazole staining is based on differential salt binding. The affinity between the divalent cations and proteins is exploited in this method. Gels were fixed with 50% methanol and 5% acetic acid for 20 min. and then incubated with agitation in 0.2M imidazole with 0.1 % SDS for 15 min. After a brief rinse in deionized water, it was incubated with gentle agitation in 0.2M zinc sulfate solution for 30 - 60 sec. Zinc ions bind to proteins through negatively charged amino acids. Imidazole reacts with unbound zinc ions and a complex salt containing zinc, SDS and imidazole precipitates on the gel forming an opaque layer leaving protein bound zinc complexes as visible translucent bands on an opaque white background (Zn-SDS-IM complex).

Modified CBB staining

Modifications in this method of staining are inclusion of an additional fixing and sensitization step prior to staining, which makes proteins more prone to dye binding. After electrophoresis, the gel was fixed with 10 %acetic acid, 10 %methanol and 40 % ethanol for 1 h and sensitized with acetic acid 1 % and ammonium sulfate 10 % for 20 min. Staining of the gel was performed with 5% acetic acid, 45% ethanol and 0.1% CBB R 250 and destained with 3% acetic acid and 30% ethanol.

Modified Kang's CBB staining

Coomassie brilliant blue G has two additional methyl groups and has a greenish tint compared to CBB R which makes the process of staining the proteins quick with fairly good sensitivity. Addition of aluminium hydroxide and phosphoric acid to the staining solution improves the sensitivity. After electrophoresis, the gel was immersed in a solution containing 5% aluminium hydroxide, 8% phosphoric acid, 0.04% CBB G and 30% methanol for 30 min. with gentle shaking and destained with 2% phosphoric acid and 10 %methanol, until the background is clear.

Sweet silver staining

Several efforts had been taken to retain the sensitivity of the most widely employed silver staining method, while removing its deleterious effects on the proteins. In this method, formaldehyde is replaced with reducing sugars in alkaline-borate buffer in the development step of silver-nitrate method, which theoretically makes this staining method compatible with MS analysis. The electrophoresed gel was fixed with 30% ethanol and 10% acetic acid for 1 h, after which it was sensitized with 12mM sodium thiosulfate. After brief rinse in deionized water, staining was done with the solution containing 12mM silver nitrate for 30 min. Gels were agitated in developing solution containing 2% reducing sugars (galactose), 100mM boric acid, 150mM sodium hydroxide, 50µM of sodium thiosulfate for 1-2 min. Similarly, 2% glucose was used in the developing solution in Ag-glucose staining method. Reduction of the silver ions was stopped by 0.3M Tris and 2% acetic acid. Some of the gels required destaining, as the dynamic detection range of silver-ion based staining methods is limited resulting in too darker staining of abundant spots. Destaining of silver stained gels was done by chemical reduction method employing 30mM potassium ferricyanide and 100mM sodium thiosulfate.

Results and discussion

The inevitability of the application of proteomic tools in unraveling plant defense and plant-pathogen interaction is being much appreciated in the recent years (Rampitsch et al. 2006, Mehta et al. 2008). Viswanathan et al. (2010) highlighted the gaining importance of proteomics as a valuable tool to decipher host resistance against diseases in sugarcane. 2-DGE coupled with MS is one of the most suitable proteomic tools in studying the regulation of proteins in response to any biotic/abiotic stresses (Lee et al. 2006). Inspite of several glistening advantages of the proteomic tool in discussion, successful MS analysis is often hindered by the detection method employed to stain 2D gels. The present study envisaged to identify proteins that are responsive to priming with different inducers (BTH and *Cf*) in order to decipher the SAR-mediated defense response in sugarcane. Proteins from the cane stalks primed with different inducers were extracted and resolved by 2-DGE as per the standardized protocol. Preliminary work carried out at the Institute indicated that silver stained protein spots when subjected to MS analysis were found to be partially compatible (Fig. 3). Subsequent analysis of the post 2D-processes indicated detained protease digestion of the proteins and peptide extraction from gel, which could be due to the formaldehyde induced cross-linking of the peptides (Shevchenko et al.1996). This in turn highlighted the need for an appropriate staining method with comparable sensitivity and better MS-compatibility.

Silver nitrate staining is regarded as the most economic method of staining proteins. However, the linearity over a wide detection range and compatibility with MS is limited. Formaldehyde induced cross-linking of the peptides was attributed for the unsuccessful MS analyses, which led to this detailed study to explore the sensitivity of the staining methods reported to be compatible with MS analyses. For evaluating the sensitivity, BSA standards (up to 10ng) resolved on 12% SDS-PAGE

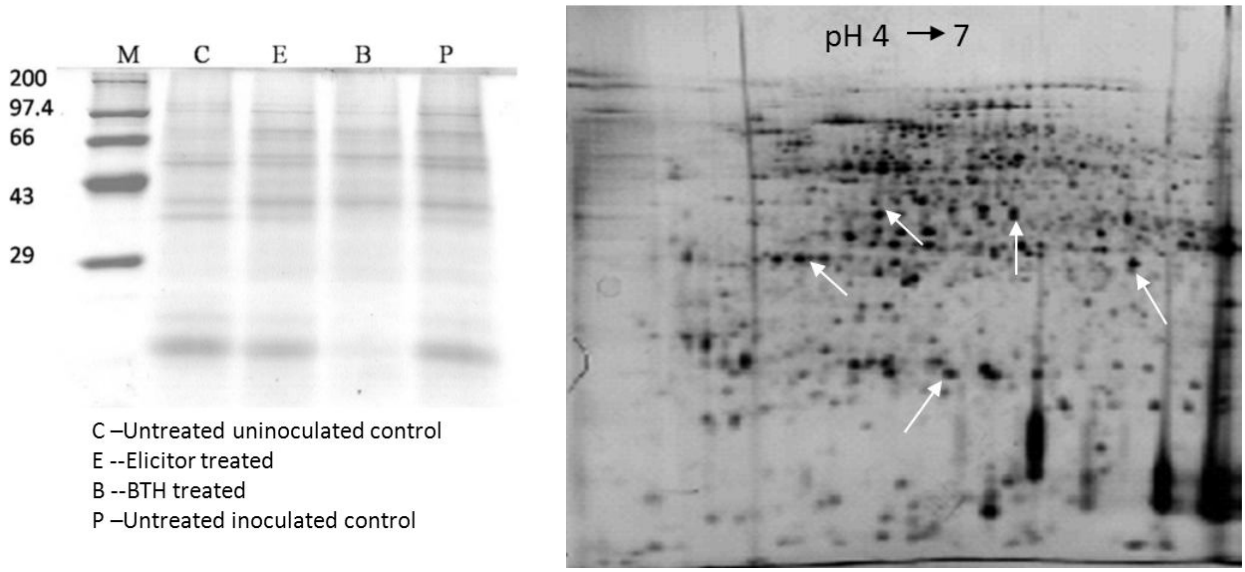

Fig. 3. 1D and 2D gel profiles

gels were stained with different methods, *viz*. coomassie brilliant blue (CBB), Zinc-Imidazole method (Zn-Im), modified coomassie brilliant blue (M-CBB), modified Kang's method or coomassie brilliant blue G staining (MK) and sweet-silver staining methods (silver-glucose (Ag-glu) and silver galactose (Ag-gal)). Gel analysis to compare the sensitivity among the methods evaluated indicated that Ag-galactose, Ag-glucose and modified Kang's method were the most sensitive staining methods (Fig. 4). The lowest detectable concentration of BSA by Ag-galactose staining was 10ng whereas 50ng of BSA was detected when galactose was replaced with glucose. However, dark background staining

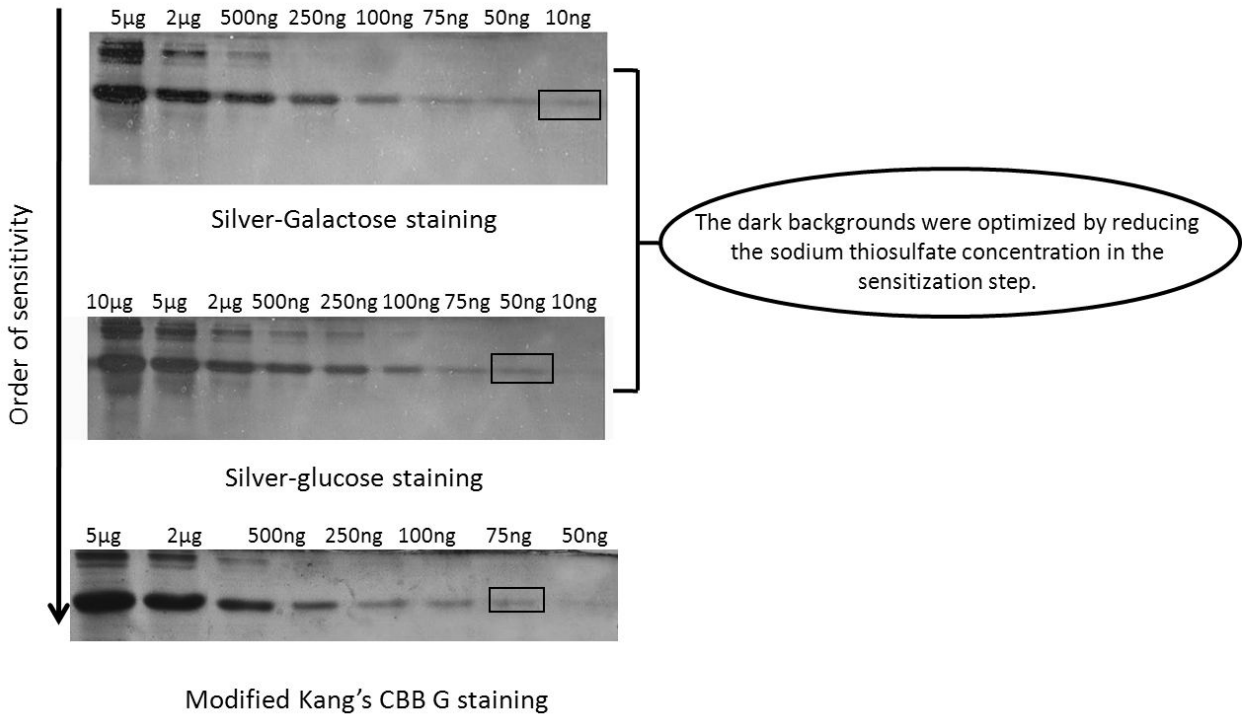

Fig. 4. SDS-PAGE profile of the most sensitive staining methods

was observed in the sweet silver staining methods. Modified Kang's method also had a comparable sensitivity; the lowest detectable concentration of BSA stained was 75ng. Sensitivity of the other methods, *viz*. coomassie brilliant blue (CBB) (300ng), Zinc-Imidazole method (Zn-Im) (1µg) and modified coomassie brilliant blue (M-CBB) (200ng) was comparatively low (Fig. 5). To conclude, results of the study to compare the relative sensitivity of different staining methods indicated that sweet silver methods (Ag-gal and Ag-glu) were most sensitive followed by modified Kang's method.

In the sweet silver methods, reducing sugars (glucose and galactose) in alkaline borate buffer served the purpose of reducing protein bound-silver ions and thus making the visibility of the protein bands better, which otherwise was contributed by formaldehyde in the conventional silver staining. A new variant of the conventional silver-nitrate staining was first described by Chevallet et al. (2008) that is completely devoid of formaldehyde. However, background of the resultant gel profiles stained with the sweet silver methods resulted in dark background staining. The situation thus emphasized the need for further optimization to improve upon the resolution. The reason for the dark background staining was speculated to be the increased sodium thiosulfate concentration (12mM in sweet silver staining) in the sensitization step of the staining protocol. Reduced concentration of sodium thiosulfate (12µM) and avoidance of the same in alkaline borate buffer (developing solution) with reducing sugars, gave comparatively lesser background staining.

In the modified Kang's method, addition of aluminium sulfate in 8% phosphoric acid could be the referable factor for such enhanced sensitivity in a dye-based protein staining method. Alhough the use of aluminium sulfate and phosphoric acid to stain proteins in polyacrylamide gels was reported by Kang et al. (2002), it was further improved upon by Mario Pink et al. (2010) by increasing the percentage of phosphoric acid to 8% (instead of 2%) and it was reasoned that the improved sensitivity was due to the increase in concentration of phosphoric acid in the staining solution. Though the method was reported to be efficient in staining protein bands as low as 2ng, for unexplained reasons the lowest stained concentration of BSA in our study was 75ng.

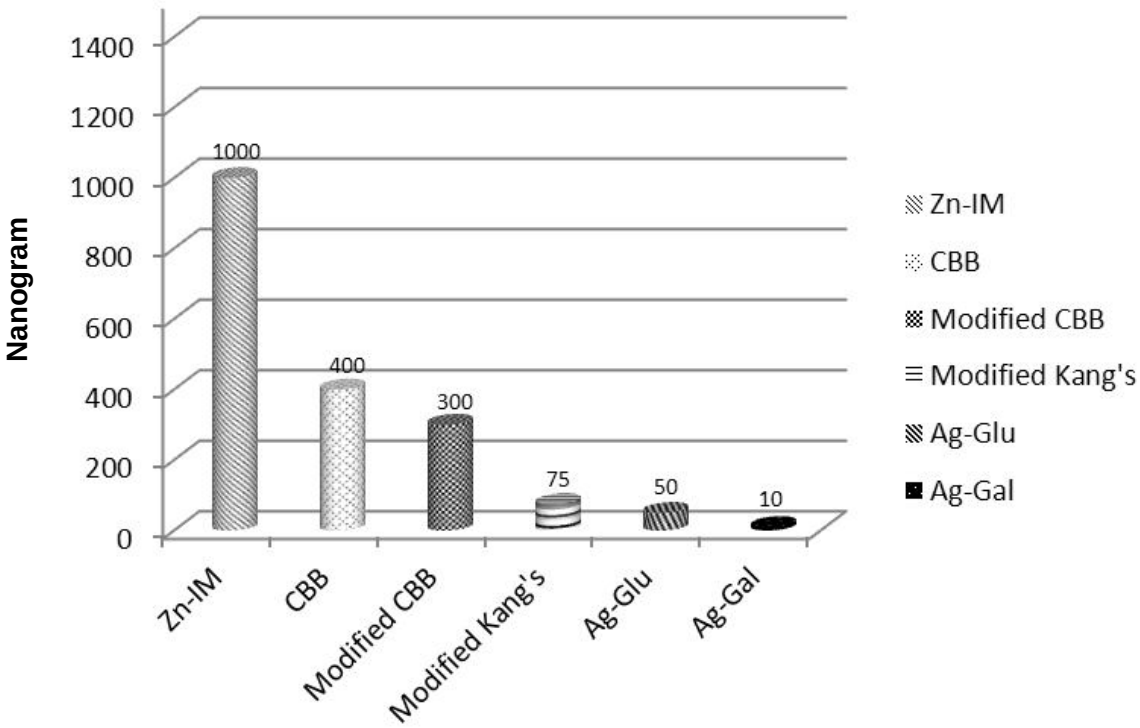

Fig. 5. Relative sensitivity of the evaluated staining methods

Sensitivity is a limitation in dye based staining methods. However, the linearity and compatibility with MS analysis is highly commended; increasing the concentration of the proteins to be resolved by 2-DGE and prolonging the staining duration might make CBB and M-CBB methods more suitable for 2-DGE. It is speculated that the low sensitivity of Zn-IM method might be improved by altering the concentrations of zinc and imidazole solutions.

Though sweet silver and modified Kang's methods were found to be the most sensitive among the methods evaluated, the MS-compatibility of these methods is being evaluated in detail before recommending the protocol as the most compatible staining method for 2-DGE. Preliminary analyses of the 2-D gel profiles of proteins from different inducer treatments indicated differential regulation of proteins probably in response to SAR-priming. The results obtained on protocol optimization would aid in subsequent identification of the differentially regulated proteins that are responsive to inducers/ elicitors. Further progress in this line of work is envisaged to unveil SAR mediated defense responses in sugarcane.

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