Proceeding Paper

Exogenous Nitric Oxide Promotes Growth and Enhances Tolerance against Drought Stress in Banana [†]

Su-Ee Lau and Boon Chin Tan *

Centre for Research in Biotechnology for Agriculture, Universiti Malaya, Kuala Lumpur 50603, Malaysia; suee@um.edu.my

* Correspondence: boonchin@um.edu.my; Tel.: +60-3-79677982

† Presented at the 2nd International Electronic Conference on Plant Sciences—10th Anniversary of Journal Plants, 1–15 December 2021; Available online: https://iecps2021.sciforum.net/.

Abstract: Drought stress is a major factor limiting crop yield. Nitric oxide (NO) is an important signal molecule that plays diverse roles in plant growth and defensive responses. However, little is known about the role of NO produced by plants under drought stress. In this study, we investigated the effects of sodium nitroprusside (SNP), an NO donor, on root growth and polyethylene glycol (PEG)-induced drought stress in bananas. Exogenous supply of NO improved root growth. We then applied a gel-based proteomic approach to investigate the root responses of bananas to define the role of NO. Ten proteins from 26 protein spots were identified using mass spectrometry. The majority of these proteins were stress-response and carbohydrate and energy metabolism-related proteins. PEG treatment significantly reduced plant growth. However, the application of SNP significantly alleviated the inhibiting effect of the drought stress in a dose-dependent manner. In contrast, the cPTIO (an NO scavenger) at a concentration equimolar to the SNP reversed the effect, suggesting that the promotive effects may be due to NO itself. These findings suggest that exogenously applied NO can appreciably improve drought tolerance in bananas, affirming its role in this stress-survival mechanism.

Keywords: abiotic stress; crop improvement; drought; nitric oxide; proteomics

1. Introduction

Environmental stresses are significant constraints to agricultural production. The water deficit caused by soil drought has been one of the main threats affecting crop growth and production worldwide [1]. Drought stress might disrupt plant cellular redox homeostasis, leading to oxidative stress and causing injury and cell death [2]. Furthermore, it would cause a profound impact on root functionality and plant growth. To survive, plants respond to drought stress by changing at molecular, morphological, biochemical, and physiological levels [3]. Therefore, it is imperative to understand the drought-responsive mechanisms in plants.

Nitric oxide (NO) is a water- and lipid-soluble biological signaling molecule induced by multiple hormonal and environmental stimuli [1]. It plays an essential role in cytoprotection by regulating the level of reactive oxygen species and toxicity [4]. Many studies have demonstrated an increased NO production in drought-stress plants, including vegetables, horticultural and woody plants, indicating that NO is involved in drought stress signaling [5,6]. However, these studies mostly rely on exogenous application of NO-donors, such as sodium nitroprusside (SNP), S-nitro-N-acetylpenicillamine (SNAP) and S-nitroglutathione (GSNO). The beneficial effects of NO depend on its location and concentration. For instance, exposure to relatively high doses of NO has been found to influence the normal metabolism and reduce respiration, leading to cytotoxicity [7]. Due to the gaps

in our knowledge on the molecular identities and biosynthetic mechanisms of NO in plants, the determination of optimal NO dosage is indispensable.

In this study, we determine the impacts of SNP on polyethylene glycol (PEG)-induced drought stress in bananas. We first evaluated the growth and proteome changes of banana roots treated with different concentrations of SNP. After determining the effective concentration of PEG to inhibit plant growth, we then analyzed the morphological changes of PEG-treated bananas or in combination with SNP and an NO scavenger.

2. Results

The effects of exogenous supplied SNP on the root growth of bananas were evaluated (Figure 1A). The medium containing 5 μM SNP was significantly different compared to other treatments as it induced the highest number of roots (8 roots per shoots) with the most increased mean root length of 13.2 cm (Figure 1B,C). The root growth was reduced with higher concentrations of SNP (>10 μM). To identify the protein changes of SNP-treated bananas, the total protein from the untreated root (MSO) and root treated with 5 μM SNP were extracted for two-dimensional gel electrophoresis and mass spectrometry analysis (Figure 1D,E). On the gel maps, 210 and 313 protein spots were detected for MSO and SNP-treated extracts, respectively. In total, 26 different protein spots showed significantly ($p < 0.01$) differential expression. Among these, 10 proteins were identified (Table S1).
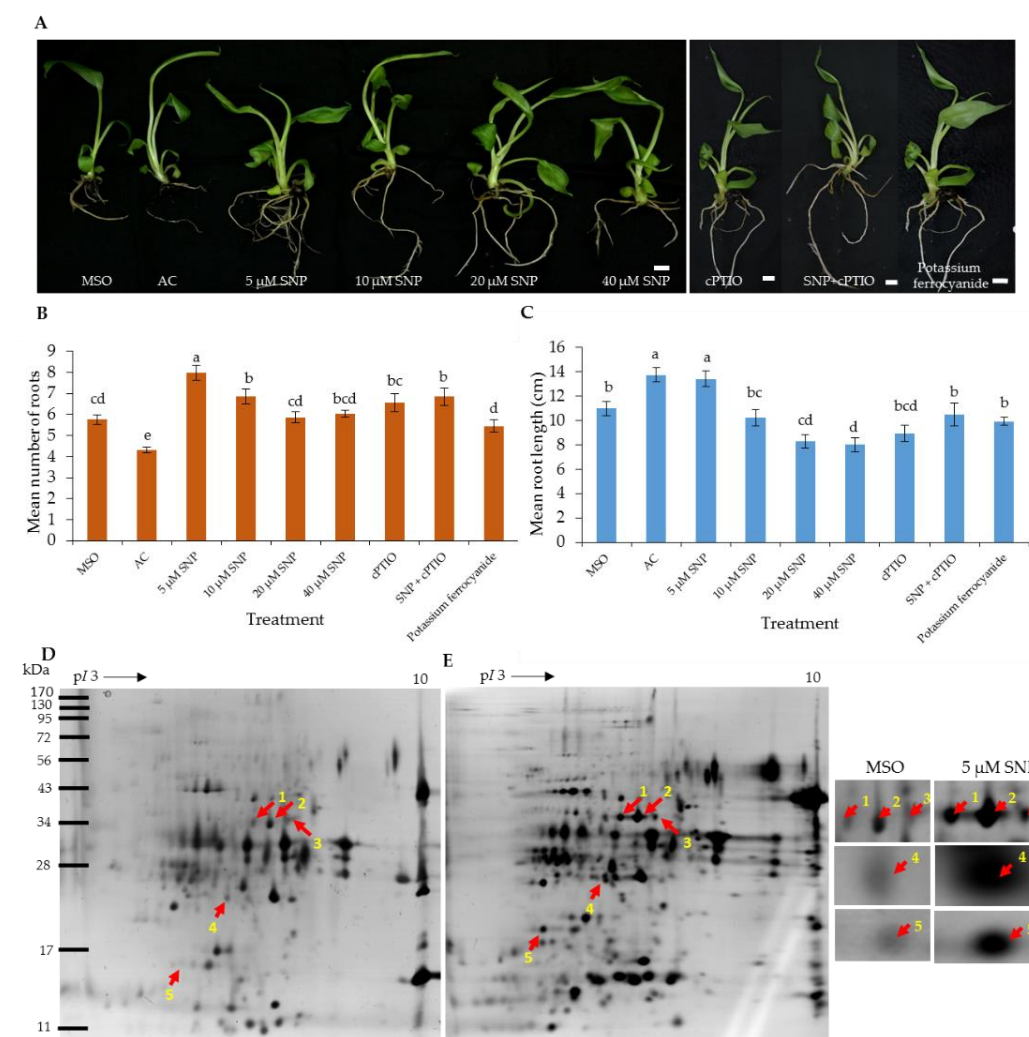

Figure 1. Influence of sodium nitroprusside (SNP) on banana. (**A**) In vitro banana plants were grown in culture media alone (MSO) or containing activated charcoal (AC), different concentrations of

SNP, cPTIO alone, cPTIO and SNP, or potassium ferrocyanide after 4 weeks of culture. Bar = 1 cm. (**B**) Mean number of roots and (**C**) mean root length were recorded after 4 weeks of culture. Data are shown as the means ± SE from 30 independent biological replicates. Means indicated with the different letters were significantly different based on analysis of variance (ANOVA) followed by Duncan's multiple range test at $p < 0.05$. Protein spots were obtained from (**D**) MSO and (**E**) 5 µM SNP. Representative 2-DE gels from five biological replicates are shown.

Banana plantlets were cultured in media containing different concentrations of PEG. In general, the increased concentrations of PEG reduced the shoot and root growth (Figure 2). The leaves of bananas showed mild yellowing when incubated on media containing 2% or higher concentrations of PEG. All in vitro banana plantlets did not survive at higher concentrations of PEG (>4%) (data not shown). Based on these results, 4% PEG was selected to determine the effects of SNP on PEG-induced drought stress in bananas. PEG treatment significantly reduced plant growth (Figure 3). However, the application of SNP significantly alleviated the inhibiting effect of the drought stress. In contrast, the cPTIO at a concentration equimolar to the SNP reversed the effect, suggesting that the promotive effects may be due to NO itself.
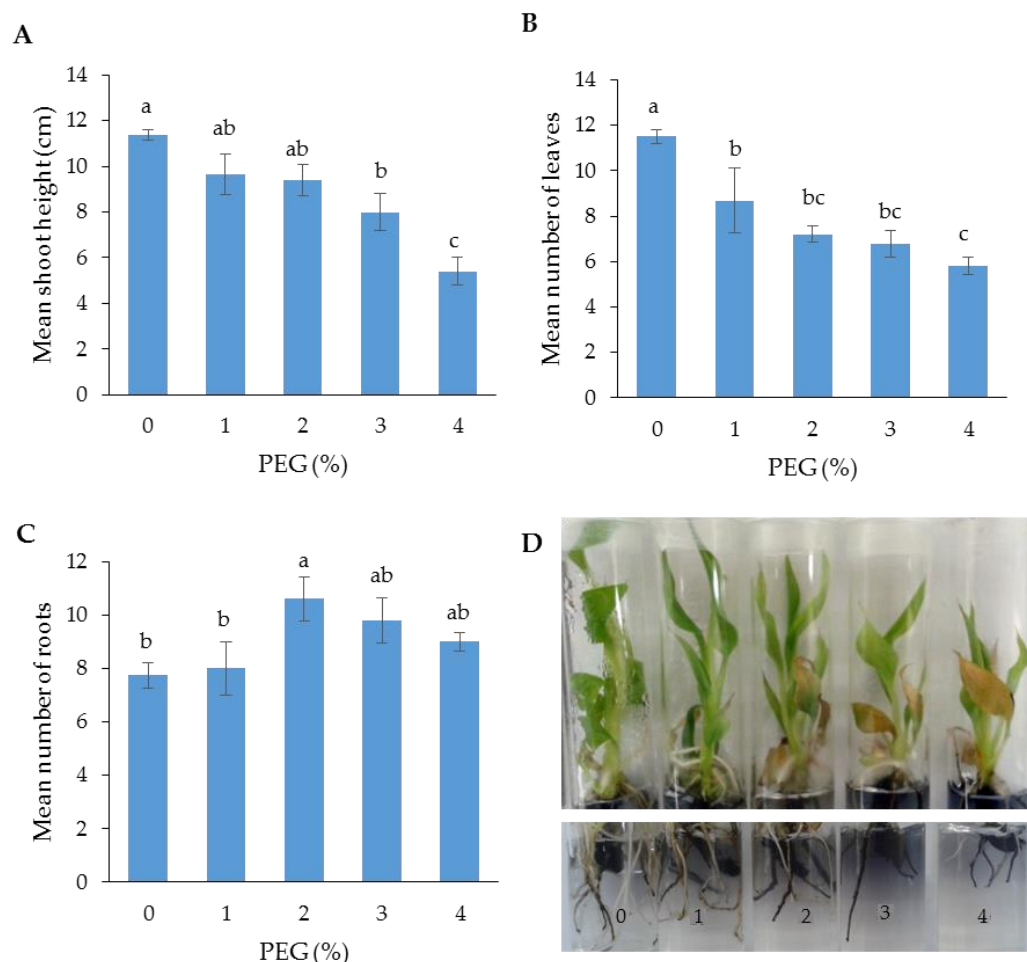
Figure 2. The morphological changes of banana plantlets treated with or without different concentrations of polyethylene glycol (PEG). (**A**) Mean shoot height, (**B**) mean number of leaves, and (**C**) mean number of roots were recorded. Data are shown as the means of 30 replicates harvested after 8 weeks of culture. Means denoted by different letters show significant differences at $p < 0.05$ according to Duncan's multiple range test. (**D**) Photographs of banana plantlets cultured in MS media containing different concentrations of PEG (%) were taken after 8 weeks of culture. Bar = 1 cm.
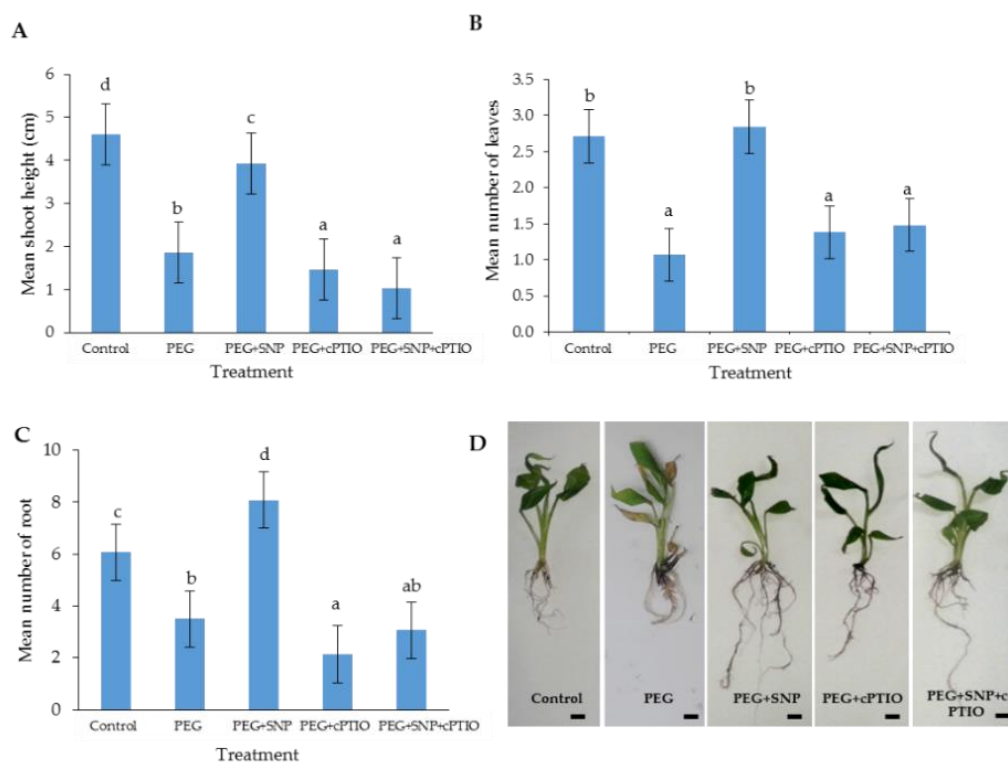
Figure 3. The morphological changes of banana plantlets treated with polyethylene glycol (PEG) with or without sodium nitroprusside (SNP) or cPTIO. (**A**) Mean shoot height, (**B**) mean number of leaves, and (**C**) mean number of roots were recorded after 8 weeks of culture. Data are shown as the means ± SE from 30 independent biological replicates. The means indicated with the different letters were significantly different based on analysis of variance (ANOVA) followed by Duncan's multiple range test at $p < 0.05$. (**D**) Photographs of banana plantlets were taken after 8 weeks of culture. Bar = 1 cm.

3. Discussion

Roots are critical organs for nutrient uptake and water absorption. Therefore, it is of great significance to explore exogenous substances that could improve plant root growth. The current study demonstrated the stimulatory effect of SNP in root growth. Exogenous supply of NO has been shown to improve root growth [8,9]. However, SNP promoted the number and length of roots in a dose-dependent manner: high levels of NO decreases root growth, but low levels of NO promotes it. In contrast, the cPTIO at a concentration equimolar to the SNP reversed the effect, suggesting that the promotive effects may be due to NO itself. However, the mechanism of NO-mediated signaling in plants is poorly understood.

A gel-based proteomics approach was used to investigate the protein changes of SNP-treated bananas. Proteins involved in carbohydrate and energy metabolisms and stress-response, such as malate dehydrogenase and triosephosphate isomerase, were increased in abundance. This might be due to energy demand caused by rapid cell growth. Certainly, better coverage of peptides and thus a full coverage of the expressed proteins, such as the gel-free proteomics approach, should be performed to draw a firm conclusion in this regard.

The shoot and root growth of PEG-treated bananas were strongly repressed. In contrast, the SNP application to the PEG-treated banana increased the shoot height and number of leaves and roots. Previous studies showed that NO promotes more roots than untreated plants, such as cucumber [10] and rice [11]. These findings suggest that SNP mitigated deleterious effects of PEG-induced stress by enhancing root formation.

4. Materials and Methods

4.1. Plant Materials and Treatment

In vitro banana plantlets (Musa acuminata cv. Berangan) purchased from the Felda Global Holding, Bandar Baru Enstek, Malaysia, were transferred to Murashige and Skoog (MS) medium supplemented with different concentrations of SNP or 8 g/L activated charcoal, 5 µM 2-(4-carboxyphenyl)-4,4,4,5,5,-tetra-methyllimidazoline-1-oxyl-3-oxide (cPTIO; an NO scavenger) and 5 µM potassium ferrocyanide (a structural analog of NO donor that does not release NO). SNP was prepared fresh. The pH of media was adjusted to 5.8. The optimal concentration of SNP was used for the subsequent PEG treatment. To mimic drought stress conditions, PEG6000-infused plates were prepared by overlaying plates containing MS medium with PEG solution. The experiments were conducted with a total of 10 plantlets per treatment and were repeated thrice. All cultures were incubated at 25 ± 2 °C under a 16-h light and 8-h dark cycle.

4.2. Protein Extraction

The harvested root samples were ground into fine powder in the presence of liquid nitrogen. Total proteins were extracted according to Tan et al. [12]. The extracted proteins were resolubilized in IPG buffer (7 M urea, 2 M thiourea, 4% (*w/v*) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 12 µL/mL DeStreak (GE Healthcare, USA), 0.2% (*v/v*) Bio-Lyte pH 3–10). The extract was sonicated for 15 min in a water bath sonicator and incubated with 10 µL/mL nuclease mix (GE Healthcare, USA) for 30 min at room temperature. After centrifugation, the supernatant was kept at −80 °C until use. The quantification of proteins was carried out using the Bradford method.

4.3. Two-Dimensional Gel Electrophoresis

Isoelectric focusing was performed on 7 cm IPG strips (pH 3–10) (Bio-Rad, USA). Strips were rehydrated overnight in 7 M urea and 2 M thiourea containing 4% CHAPS, 12 µL/mL DeStreak, 0.2% Bio-Lyte (pH 3–10), bromophenol blue and the protein extracts. IEF was performed using a Protean IEF Cell (Bio-Rad, USA) at 20 °C, following the conditions described by Tan et al. [13]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was conducted using a Mini Protean Tetra Cell apparatus (Bio-Rad, USA).

4.4. Detection of Proteins

The gels were stained according to Yan et al. [14], digitized using a GS-800 Densitometer and analyzed using Quantity One and PDQuest 2-D Analysis software version 8.0.1 (Bio-Rad, USA). After matching, gel images were normalized using the local regression model algorithm and the relative change in protein abundance for each protein spot was calculated. Differentially altered proteins were based on greater than two-fold changes between the average expression levels in groups of three representative replicate gels at the probability level of $p < 0.01$, determined by the Student's *t*-test.

4.5. Digestion of Proteins and Protein Identification

Proteins excised from 2-D gels were enzymatically digested into peptides using trypsin, according to Bringans et al. [15]. Pooled extracted peptides were dried (Speedvac, Eppendorf, USA) and sent to Proteomics International Pty Ltd. (Australia) for matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF-TOF) tandem mass spectrometer analysis. Tandem MS data were used to identify proteins of interest using Mascot search software (Matrix Science, UK) to interrogate the Ludwig NR database (Ludwig Institute, Australia). The search parameters are as follows: taxonomy restricted to Viridiplantae, one missed trypsin cleavage, peptide tolerance of 0.4 Da, MS/MS tolerance of 0.4 Da, peptide charge of +1 (monoisotopic), cysteine carbamidomethylation as a fixed modification and methionine oxidation as a variable modification. The identification was based on the Mascot score, maximum peptide coverage of the protein sequence and the

additional experimental confirmation of observed molecular mass and isoelectric focusing point of each protein. Gene ontology analysis for individual proteins was performed by searching the protein knowledgebase (UniProtKB) to reveal the biological functions of identified proteins.

4.6. Statistical Analysis

The data were recorded and analyzed statistically by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at a significance level of $p < 0.05$.

References

1. Lau, S.-E.; Hamdan, M.F.; Pua, T.-L.; Saidi, N.B.; Tan, B.C. Plant nitric oxide signaling under drought stress. *Plants* **2021**, *10*, 360. https://doi.org/10.3390/plants10020360.
2. Amnan, M.A.M.; Pua, T.-L.; Lau, S.-E.; Tan, B.C.; Yamaguchi, H.; Hitachi, K.; Tsuchida, K.; Komatsu, S. Osmotic stress in banana is relieved by exogenous nitric oxide. *PeerJ* **2021**, *9*, e10879, doi:10.7717/peerj.10879.
3. Phookaew, P.; Netrphan, S.; Sojikul, P.; Narangajavana, J. Involvement of miR164- and miR167-mediated target gene expressions in responses to water deficit in cassava. *Biol. Plant.* **2014**, *58*, 469–478.
4. Wani, K.I.; Naeem, M.; Castroverde, C.D.M.; Kalaji, H.M.; Albaqami, M.; Aftab, T. Molecular mechanisms of nitric oxide (NO) signaling and reactive oxygen species (ROS) homeostasis during abiotic stresses in plants. *Int. J. Mol. Sci.* **2021**, *22*, 9656. https://doi.org/10.3390/ijms22179656.
5. Xiong, J.; Zhang, L.; Fu, G.; Yang, Y.; Zhu, C.; Tao, L. Drought-induced proline accumulation is uninvolved with increased nitric oxide, which alleviates drought stress by decreasing transpiration in rice. *J. Plant Res.* **2012**, *125*, 155–164.
6. Cai, W.; Liu, W.; Wang, W.S.; Fu, Z.W.; Han, T.T.; Lu, Y.T. Overexpression of rat neurons nitric oxide synthase in rice enhances drought and salt tolerance. *PLoS ONE* **2015**, *10*, e0131599.
7. Santisree, P.; Bhatnagar-Mathur, P.; Sharma, K.K. NO to drought-multifunctional role of nitric oxide in plant drought: Do we have all the answers? *Plant Sci.* **2015**, *239*, 44–55.
8. Tan, B.C.; Chin, C.F.; Alderson, P. Effects of sodium nitroprusside on shoot multiplication and regeneration of *Vanilla planifolia* Andrews. *Plant Cell Tissue Organ Cult.* **2013**, *105*, 457–463.
9. Li, Y.; Wu, Y.; Liao, W.; Hu, L.; Dawuda, M.M.; Jin, X.; Tang, Z.; Yang, J.; Yu, J. Nitric oxide is involved in the brassinolide-induced adventitious root development in cucumber. *BMC Plant Biol.* **2020**, *20*, 102. https://doi.org/10.1186/s12870-020-2320-y.
10. Xu, X.T.; Jin, X.; Liao, W.B.; Dawuda, M.M.; Li, X.P.; Wang, M.; Niu, L.J.; Ren, P.J.; Zhu, Y.C. Nitric oxide is involved in ethylene-induced adventitious root development in cucumber (*Cucumis sativus* L.) explants. *Sci. Hort.* **2017**, *215*, 65–71.
11. Kushwaha, B.K.; Singh, A.; Tripathi, D.K.; Sharma, S.; Prasad, S.M.; Chauhan, D.K.; Kumar, V.; Singh, V.P. New adventitious root formation and primary root biomass accumulation are regulated by nitric oxide and reactive oxygen species in rice seedlings under arsenate stress. *J. Hazard. Mater.* **2019**, *361*, 134–140.
12. Tan, B.C.; Chin, C.F.; Liddell, S.; Alderson, P. Proteomic analysis of callus development in *Vanilla planifolia* Andrews. *Plant Mol. Biol. Rep.* **2013**, *31*, 1220–1229. https://doi.org/10.1007/s11105-013-0590-3.
13. Tan, B.C.; Chin, C.F.; Liddell, S.; Alderson, P. Identified proteins in roots of *Musa acuminata* cv. Berangan. *Minerva Biotecnol.* **2014**, *26*, 115–126.
14. Yan, J.X.; Wait, R.; Berkelman, T.; Harry, R.A.; Westbrook, J.A.; Wheeler, C.H.; Dunn, M.J. A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. *Electrophoresis* **2000**, *21*, 3666–3672.
15. Bringans, S.; Eriksen, S.; Kendrick, T.; Gopalakrishnakone, P.; Livk, A.; Lock, R.; Lipscombe, R. Proteomic analysis of the venom of *Heterometrus longimanus* (Asian black scorpion). *Proteomics* **2008**, *8*, 1081–1096.