Plasma Technology and Abiotic Elicitor Effectively Increased Isothiocyanates, Bioactive Compounds and Cytotoxicity against Caco2 Cells in Mustard Green Microgreen Extract

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Abstract:

The objective of this study was to study the effect of plasma technology and abiotic elicitor on percent germination, length of stem, fresh weight, total isothiocyanate, bioactive compounds and cytotoxicity against Caco2 cells of mustard green (*Brassica juncea* L. Czern. et Coss) microgreens germinated on vermiculite at 25 °C in 12 h light/12 dark cycle for 7 days. The results showed that plasma treatment and abiotic elicitor including sucrose, NaCl and CaCl2 did not have any effect on the % germination, but it affected the length of mustard green stems. Using only plasma treatment was able to increase the total isothiocyanate content (2.65 mmol/100g DW), total phenolic content (5.08 mg GAE/g DW), total flavonoid content (0.17 mg RE/g DW), antioxidant activity by DPPH assay (7.93 mg Trolox /g DW) and FRAP assay (15.42 mg Fe²⁺/g DW). The lowest IC50 of mustard green extract against CaCo2 cells was found when incubated for 72 h at 30.61 µg/mL. Treated Caco2 cells showed signs of shrinkage and membrane blebbing i.e. apoptosis. This study indicated that using plasma technology can improve the physiology of mustard microgreens and enhance the isothiocyanate content, bioactive compounds and cytotoxicity. Therefore, this technology can be used for improving the nutritive value and phytochemicals of microgreens as functional foods.

Keywords: Isothiocyanate, Cytotoxicity, Mustard green, Plasma, Elicitor

Introduction

Microgreens have recently become popular forms of vegetables and widely consumed around the world because they contain higher content of nutritive compounds such vitamins and minerals when compared to the same type of fully grown vegetables [1]. Microgreen vegetables have high nutritional value and also contain phytochemicals which exhibit antioxidant property, anti-inflammatory, reducing an ischemic heart disease, stroke, rate of illness and death rate from cancers [2][3][4]. It has been well-known that consuming vegetables in Cruciferae family has a positive effect on the metabolism and has remedial effects on various illnesses and preventing chronic diseases. Cruciferae family contains high glucosinolates (GSLs) which are precursors of isothiocyanates (ITCs). ITCs are degradation products occurred during the hydrolysis process of GSLs by myrosinase enzyme (MYR) of plant tissues or many bacteria that live in the intestine [5][6]. One of the ITC products that have been extensively studied is sulforaphane (SFN) which is a product from the hydrolysis of glucoraphanin in broccoli. Previous research reported that using plasma technology makes the radish sprouts grow faster approximately 9-12% [7]. Plasma technology causes the surface of the seeds attract more oxygen-containing molecules and makes the seeds absorb more water molecules, therefore, seeds are easier to germinate. Plasma is also a temporary source of Reactive Oxygen Species (ROS) during the treatment of seeds. It induces the metabolism of secondary metabolites by stress

such as flavonoids synthesis without destroying plant tissue [8][9]. In addition, it was found that using organic elicitors such as salt or sugar can lead to increased amount of GSLs in plants. High concentrations of salt caused physical stress on broccoli sprouts and resulted in increased activation of MYR[10]. Using high concentration of sucrose influences aliphatic GSL synthesis and anthocyanins in broccoli sprouts [11], but there is no report on using plasma technology or organic stimulants in the cultivation of local microgreens in Thailand. Therefore, this research aims to study the influence of cold plasma technology and abiotic elicitors on percentage germination, length of stem, fresh weight, ITC content and bioactive compounds of Thai mustard green microgreen and also its cytotoxicity against Caco2 cells.

Materials and Methods

Chemicals and materials

Chemicals including 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), Folin-Ciocalteu phenol reagent (FCR) and gallic acid, Benzyl isothiocyanate (BITC) were obtained from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA). Trolox standard (TE) (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 2,4,6-Tripyridyl-5-Triazine (TPTZ) were purchased from Fluka Chemicals (Buchs, Switzerland). Methanol for HPLC grade was obtained from BDH (Poole, UK). Dulbecco's modifed Eagle's medium (DMEM), Fetal bovine serum (FBS) and all other reagents used in cell culture were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). MTT (Amresco, OH, USA). Mustard green seeds were purchased from online shop (https://www.pwcmallonline.com). Human Colorectal Adenocarcinoma (CaCo2 ATCC[®] HTB-37TM) was obtained from American Type Culture Collections (ATCC, Manassas, VA, USA).

Plant material and cultivation conditions

Seed treatment with plasma technology was performed at Faculty of Engineering Srinakharinwirot University, Nakhon Nayok Province. The procedure was done according to the previous method [3]. Mustard green seeds (100 seeds/treatment) were treated with plasma at the power level of 19 kV for 5 min. After that, seeds were washed and soaked with distilled water overnight. The seeds were sowed in a layer of vermiculite in a tray at 25°C (42 μ mol/sec/m² of light concentration, 12 h light/12 h dark cycle) in plant tissue culture room. Twenty mL of the tested elicitors; mL, 176 mM sucrose, 160 mM NaCl,10 mM CaCl₂ were individually sprayed on the leaves of tested microgreens; however the control was spraying with deionized water (DI) for 7 days. Microgreens of 7-d olds were gently cut and collected. The germination percentage, length of stem and fresh weight were measured. Microgreen samples were packed in polyethylene bags and stored at -80 °C for next analysis.

Extraction and determination of ITC

Extraction of ITC followed the previous method [12]. The freeze dried microgreens (250 mg) were homogenize with 4 mL of 0.1 M citrate-phosphate buffer (pH 7.0) and incubated in shaking incubator (LSI-1005R, Lab Tech, Korea) at 37 °C for 1 h at 250 rpm. Extracted ITC with dichloromethane (DCM) in the ratio of 1:1 and shaken for 30 min at 250 rpm. The mixtures were centrifuged at 10,000 g for 5 min using centrifuge (Universal 320R, Germany). The supernatants were added with 0.5 g magnesium sulfate (MgSO₄) for removing the moisture before mixing and centrifuged at the same condition. The clear supernatants were mixed with methanol in the ratio of 1:4 for ITC determination. Determination of total ITC was performed as previously [13]. Ten microliters of sample were added in the 96-well plate and mixed with 90 μ L of methanol and 90 μ L of 0.1 M phosphate buffer (pH 8.0). After 2 min, 10 μ l of 0.08 M benzene 1,2 dithiol solution were added to the well plate and mixed using the pipette and incubated at 60 °C for 2 h. The absorbance was measured at 360 nm using microplate reader (Synergy 4HT Microplate Reader). Results were expressed as mmol equivalents in 100 g of dried sample (mmol/100 g DW). Benzene-isothiocyanate (BITC) was used as a standard.

Microgreen extraction for bioactive compounds and antioxidant activity

Dried microgreens (0.1 g) were extracted with 5 mL of 80% methanol and incubated at 37 °C on an orbital shaker at 200 rpm. After 24 h, the mixture was centrifuged at 10,000 g for 20 min and repeated

extracting twice. The supernatant was filtered through Whatman (No.1). The extracted sample was stored at -80 °C.

Determination of total phenolic content (TPC)

TPC was determined using Folin–Ciocalteu reagent according to the previous reported [14][15]. Twenty microliters of sample and 100 µL of 10% Folin-Ciocalteu reagent were pipetted into 96-well plate. After 5 min, 80 µL of 7.5% NaHCO₃ were added, mixed, and incubated at room temperature for 30 min. The reaction was measured at 750 nm using microplate reader. Gallic acid was used as a standard and the results were reported as mg gallic acid equivalent (GAE)/g DW.

Determination of total flavonoid content (TFC)

The TFC was evaluated according to the previous reported [16] with a minor modification. Microgreen extracts (20 μ L) were mixed with 60 μ L of DI water in well plate. After that, 10 μ L of 5% NaNO₃ and 10 μ L of 10% AlCl₃.6H₂O were added, mixed and left for 5 min at room temperature. The mixture was reacted by adding with 100 μ Lof 1 M NaOH, mixed and kept for 30 min before measuring at 510 nm. Rutin was used as standard and expressed as mg rutin equivalents (mg RE/g DW).

Free-radical scavenging activity on DPPH-

The 2,2- diphenyl- 1- picrylhydrazyl (DPPH) radical scavenging of microgreen extract was determined according to the previous reported [17] with some modifications. Three hundred microliter of microgreen samples or control (80% methanol) were mixed with 180 μ l of 0.2 mM of DPPH solution in methanol. After 30 min, The mixture was measured measured at 595 nm. A standard curve of trolox was used and expressed as mg trolox equivalent (mg TE/g DW).

Ferric reducing antioxidant power (FRAP)

The total reducing capacity of micreogreem extracted was evaluated using FRAP assay according to the previous reported [18] with some modifications. Working FRAP reagent was initially prepared consisting of 300 mM acetate buffer pH 3.6 by, 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl and 20 mM iron (III) chloride solution (FeCl₃) in the ratio of 1:1:10 (v/v/v), respectively, and incubated at 37 °C for 30 min before using. Microgreen extract s(20 μ L) were mixed with 180 μ L of FRAP reagent and were then incubated for 90 min at room temperature before measured at 593 nm. FRAP values were reported as mg Fe(II)/g DW.

Cell line and cell culture

The Human Colorectal Adenocarcinoma CaCo2 cancer cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained according to the recommendations of the ATCC at 37 °C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with penicillin, 100 μ L/mL of streptomycin and 10% Fetal bovine serum. DMEM media was renewed every 3 days and trypsinized with 0.05% trypsin-EDTA, and 10⁶ cells were seeded into fresh DMEM for next analysis.

Isothiocyanate extraction for cell line

Microgreen extraction for cell lines with some modifications was done according to the previous reported [19]. Fresh microgreens (50 g) were homogenized in 50 mL of 0.1 M citrate phosphate buffer, pH 7.0 at 37 °C in shaker incubator at 250 rpm for 2 h. GSLs were hydrolyzed to isothiocyanates (ITCs) form by MYR. Then, 50 mL of homogenates and 50 mL of DCM in the ratio of 1:1 were mixed together and were then dehydrated using MgSO₄. The DCM layer was filtered through Whatman No. 1 and evaporated using rotary evaporator (Buchi R-114, Switzerland) before drying using freeze dryer. The stock solution of the extract was dissolved in 1% of dimethyl sulfoxide (DMSO) for further analysis.

Cytotoxicity assay

The effect of crude extract on human CaCo2 colon cancer cell cytotoxicity was measured using the 3-(4, 5-dimethylthiazol-2)-2, 5-diphenyltetrazolium bromide (MTT) assay according to the previous

reported [20]. The density of CaCo2 cells at 5×10^3 cells/well was placed into the 96-well plate and incubated at 37°C in 5% CO₂ for 24 h. Cells were treated with crude microgreen extract with serum free medium concentration 0-250 µg/mL by serial dilutions. After the incubated at 37°C in 5% CO₂ for 24, 48 and 72 h, 20 µL of MTT reagent (5 mg/mL) were then added to each well plate and incubated at 37°C under 5% CO₂ for 4 h. The solution in the plate was replaced with 200 µL of DMSO to dissolve the formazan crystal and absorbance at 600 nm was measuredusing microplate reader. The IC₅₀ value was calculated based on graphs ofusing % cytotoxicity and concentrations of extracts.

Cell morphology

The human CaCo2 colon cancer cells were seeded into a well plate at a density 5×10^4 cells/mL. Cells were prepared in same the conditions as above. After treatment for 24 h at 37°C under 5% CO₂, cell morphology of cancer cells was monitored using a microscope at 400X.

Statistical analysis of data

Data were reported as means \pm standard deviation (SD). Statistical analysis was performed using One-way analysis of variance (ANOVA) using software SPSS (demo version). Statistically significant differences were considered at p<0.05.

Results and discussion

Effect of plasma technology and abiotic elicitor on mustard green microgreens growth

The effects of cold plasma technology and abiotic elicitor on % seed germination (Fig. 1A) and length of stem (Fig. 1B) of mustard green microgreens were not significantly different when compared with the control except the treatment with plasma plus NaCl and only NaCl led to the lowest % seed germination. Plasma plus NaCl and sucrose, and NaCl treantments led to the lowest length of stem in mustard green microgreens. A significant increase in the level of fresh weight was observed in plasma treatment (38.33 mg/microgreen) (Fig. 2A) in mustrad green compared with the control. However, after treated using plama with NaCl, plama with CaCl₂, NaCl, and CaCl₂ led to fresh weight reduction. Ling et al. [21] found that seeds treated with plasma at low temperature can differently absorbed the moisture depending on the power of plasma used. Low temperature plasma can react with the chemical structure and shell surface of the seeds and it allows the seeds to absorb the water from the outside better. Yuan et al. [22] reported that radish sprout was inhibited after receiving 50 and 100 mM of NaCl. The salinity affects the metabolic processes of the radish and causes changes in anatomy and physiology and influence on seed germination. Esfandiari et al. [10] found that the concentration of 160 mM NaCl did not effect on the weight of broccoli sprout which was similar to the study of Lee et al. [23] who mentioned that NaCl inhibited the germination of seeds. NaCl produces hydrogen peroxide [24][25][26] and calcium helps the cell wall and cell membrane to become stronger and increased membrane permeability.



Figure 1 Effect of plasma treatment and abiotic elicitor on % germination and length of stem of mustard green microgreen. (A) % gemination (B) Length of stem. Different letters above columns indicate significant differences (*p*<0.05)



Figure 2 Effect of plasma treatment and abiotic elicitor on fresh weight and total ITC of mustard green microgreen. (A) Fresh weight. (B) Total ITC. Different letters above columns indicate significant differences (*p*<0.05)

Total ITC content

The results showed significantly different ITC contents among Thai mustard green microgreens treated with plasma (2.65 mmol/100g DW) at the highest level of total ITC followed by plasma with CaCl₂, sucrose and CaCl₂, respectively (Fig. 2B). Plasma technology and abiotic elicitor increased the total ITC compared with the control, except for plasma with sucrose, plasma with NaCl and only NaCl. Yang et al. [27] reported that using CaCl₂improved the biochemical properties in GSL synthesis by enhancing the BrST5b gene (sulfotransferase 5b) and BrAOP2 gene (2-oxoglutarate-dependent dioxygenase 2) expression and 10 mM CaCl₂ increased the amount of GSLs in broccoli. Increasing amount of GSLs and increased activity of the MYR resulted in increased ITC content. Verkerk et al. [28] found many genes including BrST and BrFMOGSOX related to the GSL synthesis.

Bioactive compounds and antioxidant capacity

The results showed The TPC and TFC of mustard green microgreen were significantly increased after treatment with plasma technology and abiotic elicitor (p<0.05) when compared with the control. After treated with plasma, the highest level of TPC (5.37 mg GAE/g DW) (Fig. 3A) and TFC (1.42 mg RE/g DW) (Fig. 3B) were detected. Nevertheless, NaCl treatment led to TPC decrease.

Antioxidant activity by DPPH and FRAP assays were also significantly different in plasma treatments (p<0.05). Results found that only plasma treatment showed the highest antioxidant activity by DPPH (7.44 mg TE/ g DW) (Fig. 4A) and FRAP assay (15.42 mg FeII/g DW) (Fig. 4B). Tsai et al. [29] found that sucrose was an essential energy source for plant cells and can also add sugar to the aglycone in the process of glycosylation, which is the final reaction of the anthocyanin synthesis. Mano et al. [30] reported that flavonoids synthesis and enzymes were related to the synthesis of anthocyanins in Arabidopsis such as flavanone 3 β -hydroxylase chalcone synthase chalcone isomerase and anthocyanidin synthase. Sucrose, glucose and fructose are promoting the synthesis of GSLs and anthocyanin in cruciferous plants. Sucrose is the most effective substance to stimulate accumulation of secondary substances [31]. Researchers [32] reported that plasma stimulated gene growth regulating factors, antioxidant enzymes, and energy metabolic genes which influenced on plant growth. Reports of Bußler et al.[9] stated that plasma caused ROS and UV which induced stress in plants and thus induced plant synthesis of the secondary metabolites, antioxidant, molecules/enzymes that helped to resist those stresses.



Figure 3 TPC and TFC of mustard green microgreens. (A) TPC and (B) TFC. Different letters above columns indicate significant differences (*p*<0.05)



Figure 4 Antioxidant capacity of mustard green microgreens. (A) DPPH assay. (B) FRAP assay. Different letters above columns indicate significant differences (*p*<0.05)



Figure 5 Effects of 21 kV plasma treated mustard green microgreen on CaCo2 cell death. (A); % Cytotoxicity. (B) ; The IC₅₀ value of 21 kV plasma treated mustard green microgreen on Caco2 cells. Data represent means±SEM of three independent experiments. Different letters above columns indicate significant differences (*p*<0.05)

Cytotoxicity and cell morphology of CaCo2 cell

The principle of MTT is that the alive cancer cells have intact succinate dehydrogenase enzyme in mitochondria which turns tetrazolium salt into formazan by reduction process. The quantity of formazan is directly varied according to the number of the living cells. Cytotoxicity against CaCo2 cancer cells of crude mustard green microgreen extract treated with plasma and incubated for 24, 28 and 72 h showed the IC_{50} value of 55.89, 43.42 and 30.61 µg/mL, respectively (Fig. 5A-5B). Cells incubation for 72 h had the lowest IC_{50} indicating most effective effect.

CaCo2 cancer cells treated with crude mustard green microgreen extract at 24, 48 and 72 h (Fig. 6) showed some signs of shrinkage, membrane blebbing, organelle condensation, and fragmented cells. The severity of cells morphological changes varied with increasing concentrations of the extract. The contraction of cells and broken into small and the cell membrane aneurysm are indicators ofdead cells i.e. apoptosis. Oberhamme et al. [33] and Webb et al. [34] pointed out that the characteristic of cells death caused by apoptosis consisted of cell shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing and apoptotic bodies. Apoptosis cell death can occur via two routes, either extrinsic (activation of death receptors) or intrinsic (mitochondrial-mediated) pathways.



Figure 6 Effect of mustard green microgreens treated with plasma on cell morphology of CaCo2 cancer cells at 24, 48 and 72 h of cell incubation.

Conclusion

The plasma technology and abiotic elicitor or only abiotic elicitor have no effect on the percentage of germination in mustard green seeds. Plasma treatment affected the length of stem and the fresh weight of mustard green microgreens. It can induced ITC production and increase the bioactive compounds, antioxidant activity and cytotoxicity against CaCo2 cancer cells. This is the first report of using cold plasma technology and abiotic elicitor for Thai local vegetable microgreen cultivation. Plasma technology and abiotic elicitor can be used to improve nutrients and bioactive compounds in other plants.

Acknowledgements

This research was funded by National Research Council of Thailand (Year 2019) through Master students funding awarded to WS. Authors would like to thank Department of Biotechnology, Mahasarakham University and Central Laboratory for laboratory facilities.

References

- [1] Xiao, Z., Lester, G. E., Luo, Y., & Wang, Q. (2012). Assessment of vitamin and carotenoid concentrations of emerging food products: Edible microgreens. *Journal of Agricultural and Food Chemistry*, 60(31), 7644–7651.
- [2] Barillari, J., Cervellati, R., Paolini, M., Tatibouet, A., Rollin, P., & Iori, R. (2005). Isolation of 4methylthio-3-butenyl glucosinolate from Raphanus sativus sprouts (kaiware daikon) and its redox properties. *Journal of Agricultural and Food Chemistry*, 53(26), 9890–9896.
- [3] Ciska, E., Honke, J., & Kozlowska, H. (2008). Effect of light conditions on the contents of glucosinolates in germinating seeds of white mustard, red radish, white radish, and rapeseed. *Journal* of Agricultural and Food Chemistry, 56(19), 9087–9093
- [4] Martinez-Villaluenga, C., Frias, J., Gulewicz, P., Gulewicz, K., & Vidal-Valverde, C. (2008). Food safety evaluation of broccoli and radish sprouts. *Food and Chemical Toxicology*, 46(5), 1635–1644
- [5] Andreasson, E., Jorgensen, L.B., Hoglund, A.S., & Meijer, J. (2001). Different myrosinase and idioblast distribution in Arabidopsis and Brassica napus. *Plant Physiology*, 127(4), 1750–1763
- [6] Fahey, J. W., Salcmann, A. T., & Talalay, P. (2001). The chemical diversity and distribution of glycosinolates and isothiocyanates among plants. *Phytochemistry*, 56(1), 5–51.
- [7] Matra, K. (2016). Non-thermal Plasma for Germination Enhancement of Radish Seeds. Procedia Computer Science, 86, 132–135.
- [8] Baier, M., Foerster, J., Schnabel, U., Knorr, D., Ehlbeck, J., Herppich, W.B., et al. (2013). Direct nonthermal plasma treatment for the sanitation of fresh corn salad leaves. Evaluation of physical and physiological effects and antimicrobial efficacy. *Postharvest Biology and Technology*, 84, 81–87.
- [9] Bußler S., Herppich WB, Neugart S, Schreiner M, Ehlbeck J, Rohn S and Schlüter O. (2015). Impact of cold atmospheric pressure plasma on physiology and flavonol glycoside profile of peas ('Salamanca'). *Food Research International*, 76, 132-141.
- [10] Esfandiari, A., Saei, A., Mckenzie, M. J., Matich, A. J., Babalar, M., & Hunter, D. A. (2017). Plant Physiology and Biochemistry Preferentially enhancing anti-cancer isothiocyanates over glucosinolates in broccoli sprouts : How NaCl and salicylic acid affect their formation. *Plant Physiology et Biochemistry*, 115, 343–353.
- [11] Guo, R., Yuan, G., & Wang, Q. (2011). Sucrose enhances the accumulation of anthocyanins and glucosinolates in broccoli sprouts. *Food Chemistry*, 129(3), 1080–1087.
- [12] Luang-In, V., Deeseenthum, S., Udomwong, P., Saengha, W., & Gregori, M. (2018). Formation of sulforaphane and iberin products from Thai cabbage fermented by myrosinase-positive bacteria. *Molecules*, 23(4), 3–5.
- [13] Amron, N. A., and Konsue, N. (2018). Antioxidant capacity and nitrosation inhibition of cruciferous vegetable extracts. *International Food Research Journal*, 25(1): 65 73
- [14] Radošević, K., Srček, V.G., Bubalo, M.C., Rimac Brnčić, S., Takács, K., & Redovniković, I.R. (2017). Assessment of glucosinolates, antioxidative and antiproliferative activity of broccoli and collard extracts. *Journal of Food Composition and Analysis*, 61, 59-66.
- [15] Thomas, M., Badr, A., Desjardins, Y., Gosselin, A., & Angers, P. (2018). Characterization of industrial broccoli discards (Brassica oleracea var. italica) for their glucosinolate, polyphenol and flavonoid contents using UPLC MS/MS and spectrophotometric methods. *Food Chemistry*.

- [16] Tian, M., Xu, X., Liu, Y., Xie, L., & Pan, S. (2016). Effect of Se treatment on glucosinolate metabolism and health-promoting compounds in the broccoli sprouts of three cultivars. *Food Chemistry*, 190, 374– 380.
- [17] Zhang, L., Tu, Z.C., Yuan, T., Wang, H., Xie, X., & Fu, Z.F. (2016). Antioxidants and alpha-glucosidase inhibitors from Ipomoea batatas leaves identified by bioassay-guided approach and structure-activity relationships. *Food Chemistry*. 61-67
- [18] Wei, J., Miao, H., & Wang, Q. (2011). Scientia Horticulturae Effect of glucose on glucosinolates , antioxidants and metabolic enzymes in Brassica sprouts. *Scientia Horticulturae*, 129(4), 535–540.
- [19] Pocasap, P., Weerapreeyakul, N., & Barusrux, S. (2013). Cancer preventive effect of Thai rat-tailed radish (Raphanus sativus L. var. caudatus Alef). *Journal of Functional Foods*, 5(3), 1372–1381.
- [20] Siddiqui, M. A., Wahab, R., Ahmad, J., Farshori, N. N., Musarrat, J., & Al-khedhairy, A. A. (2017). Evaluation of cytotoxic responses of raw and functionalized multi-walled carbon nanotubes in human breast cancer (MCF-7) cells. *Vaccum*, 146, 578–585.
- [21] Ling, L., Jiafeng, J., Jiangang, L., Minchong, S., Xin, H., Hanliang, S., & Yuanhua, D. (2015). Effects of cold plasma treatment on seed germination and seedling growth of soybean. *Scientific Reports*, 4(1), 5859.
- [22] Yuan, G., Wang, X., Guo, R., & Wang, Q. (2010). Effect of salt stress on phenolic compounds, glucosinolates, myrosinase and antioxidant activity in radish sprouts. *Food Chemistry*, 121(4), 1014– 1019.
- [23] Lee, S., Kim, S.G., Park, C.M., 2010. Salicylic acid promotes seed germination under high salinity by modulating antioxidant activity in Arabidopsis. *New Phytol*.188, 626e637.
- [24] Zhang, D. H., Wadsworth, P., & Hepler, P. K. (1992). Modulation of anaphase spindle microtubule structure in stamen hair cells of Tradescantia by calcium and related agents. *Journal of Cell Science*,102(1), 79-89.
- [25] Harmon, A. C., Gribskov, M., Gubrium, E., & Harper, J. F. (2001). The CDPK superfamily of protein kinases. *New Phytologist*, 151(1), 175-183.
- [26] Zhang, L., & Lu, Y. T. (2003). Calmodulin-binding protein kinases in plants. *Trends in plant science*,8(3),123-127.
- [27] Yang, R., Hui, Q., Gu, Z., & Zhou, Y. (2016). Effects of CaCl₂ on the metabolism of glucosinolates and the formation of isothiocyanates as well as the antioxidant. *Journal of Functional Foods*, 24, 156–163.
- [28] Verkerk, R., Schreiner, M., Krumbein, A., Ciska, E., Holst, B., Rowland, I., Dekker, M. (2009). Glucosinolates in Brassica vegetables: The influence of the food supply chain on intake, bioavailability and human health. *Molecular Nutrition and Food Research*, 53(SUPPL. 2), 219–265.
- [29] Tsai, P.J., Delva, L., Yu, T.Y., Huang, Y.T. & Dufosse, L. (2005). Effect of sucrose on the anthocyanin and antioxidant capacity of mulberry extract during high temperature heating. *Food Research International*, 38, 1059-1065
- [30] Mano, H., Ogasawara, F., Sato, K., Higo, H. & Minobe, Y. (2007). Isolation of a regulatory gene of anthocyanin biosynthesis in tuberous roots of purple-fleshed sweet potato. *Plant Physiology*, 143, 1252-1268.
- [31] Koornneef, M., & Smeekens, S. (2005). Sucrose-Specific Induction of Anthocyanin Biosynthesis in Arabidopsis Requires the MYB75 / PAP1 Gene 1. *Society*, 139(December), 1840–1852.
- [32] Zhang, J. J., Jo, J. O., Huynh, D. L., Mongre, R. K., & Ghosh, M. (2017). Growth-inducing effects of argon plasma on soybean sprouts via the regulation of demethylation levels of energy metabolismrelated genes. *Nature Publishing Group*, (January), 1–12.
- [33] Oberhammer F, Wilson JW, Dive C, et al. Apoptotic death in epithelial cells: cleavage of DNA to 300 and /or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J 1993; 12:3679-84.
- [34] Webb SJ, Harrison DJ, and Wyllie AH. Apoptosis: an overview of the process and its relevance in disease. Adv in Pharmacol 1997; 41: 1-33.