Elicitation of Phenolic Biosynthesis and Antioxidative Capacities in Common Bean (*Phaseolus vulgaris*) Sprouts

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ABSTRACT

Apart from providing basic nutrients, foods also impact additional health benefits such as antioxidative, anti-cancer, antimicrobial and antidiabetic effects due to the presence of functional molecules such as phenolic compounds. Over the years, manipulation of phenolic biosynthesis has been achieved with techniques such as plant tissue cultures and genetic engineering. However, these techniques have limitations such as low consumer acceptance, low yield, plant cellular damage and genetic instability. Thus, there is need for appropriate and consumer acceptable techniques to enhance the yield of phenolic compounds in foods. Synthesis of secondary metabolites such as phenolic compounds could be elicited through the application of appropriate elicitors/stressors. For the first time, this study investigated the influence of chemical, ultrasonication and thermal elicitations of the phenylpropanoid pathway through the induction of stress at different sprouting stages of common bean (*Phaseolus vulgaris*). With each elicitation, accumulation of stress markers (H_2O_2, catalase, guaiacol peroxidase), activities of phenylpropanoid triggering enzymes (phenylalanine ammonia-lyase, tyrosine ammonia-lyase), phenolic compounds, antioxidant capacities and sprout morphological characteristics were monitored at different sprouting stages.

Among the chemical elicitors, 7 mM glutamic acid elicited the highest levels of H_2O_2 (0.12 mol/g) at 96 h of sprouting time, whereas the maximum activities of catalase (23.2 U/mg protein) and peroxidase (3.48 U/mg protein) were elicited with 5 mM glutamic acid compared to control. The 5 mM glutamic acid most significantly enhanced the activities of phenylalanine ammonia-lyase and tyrosine ammonia-lyase leading to 65% enhancements of total phenolic acids and 2.97 fold total flavonoids, and significantly enhanced DPPH, ABTS and Fe^{2+} antioxidant capacities. The chemical elicitors did not inhibit sprout's morphological characteristics.

Ultrasonication studies indicated the highest levels of H_2O_2 (0.35 mol/g), catalase (26.04 U/mg protein) and guaiacol peroxidase (8.80 U/mg protein) with 360 W (60 min) at 96 h of sprouting. Elevated stress markers observed with ultrasonic treatments enhanced activities of phenylalanine ammonia-lyase and tyrosine ammonia-lyase, resulting in enhanced total contents of phenolic acids, flavonoids and anthocyanins by 11.7, 6.6 and 11.5 folds, respectively, compared to the control. Additionally, phenolic compounds elicited with ultrasonic treatments significantly improved all tested antioxidant capacities. Furthermore, elevated stress markers stimulated with 360 W (60 min) ultrasonic treatment reduced sprouting time by 60 h, compared to control treatments, as well as enhanced radical, hypocotyl and vigor index of common bean sprouts.

Also, thermal treatments (25-control, 30, 35 and 40 °C) significantly enhanced biosynthesis of phenolic compounds. The highest accumulations of H_2O_2 and malondialdehyde were elicited with 40°C, whereas activities of catalase and guaiacol peroxidase were maximally elicited with 30°C at 96 h of sprouting. Phenylalanine ammonia-lyase was optimally elicited with 30°C (11 folds higher than control), whereas tyrosine ammonia-lyase showed a 97.4 % increase. These conditions simultaneously resulted in significant increase of phenolic compounds and antioxidant capacities. Total phenolic acids were enhanced by 7.75 times, total flavonoids by 69.3 % and total anthocyanins content by 2.29 folds, compared to control. In addition, elevated stress markers associated with 30°C treatments were non-lethal against morphological characteristics of sprouts, compared to other investigated temperature levels. For kinetic studies, accumulation rates, k (h^{-1}), estimated for H_2O_2 and malondialdehyde showed an increasing trend with temperature rise. However, activation rates observed for catalase, guaiacol peroxidase and phenylpropanoid triggering enzymes were highest at 30°C. Furthermore, activation energy

(E_a-kJ/mol) values estimated for total phenolic acids, flavonoids and anthocyanins were 30.40, 63.98 and 208.63 kJ/mol, respectively, with this result synchronizing with the high accumulation rates, k (h^{-1}), estimated for phenolic acids and flavonoids, compared to anthocyanins.

In conclusion, ultrasonic treatment of 360 W (60 min) at 96 h of sprouting elicited phenolic compounds with maximum antioxidant capacities, when compared with other investigated treatments. Thus, controlled elicitation can serve as alternative bioprocessing techniques for production of natural functional foods.

Résumé

En plus de fournir des nutriments de base, les aliments ont également un impact sur d'autres avantages pour la santé tels que les effets antioxydants, anticancéreux, antimicrobiens et antidiabétiques dus à la présence de molécules fonctionnelles comme les composés phénoliques. Au fil des ans, la manipulation de la biosynthèse phénolique a été réalisée à l'aide de techniques telles que les cultures de tissus végétaux et le génie génétique. Cependant, ces techniques ont des limites telles que la faible acceptation par le consommateur, le faible rendement, les dommages cellulaires des plantes et l'instabilité génétique. Il faut donc des techniques appropriées et acceptables pour le consommateur afin d'améliorer le rendement des composés phénoliques dans les aliments. La synthèse de métabolites secondaires comme les composés phénoliques pourrait être provoquée par l'application d'éliciteurs/stresseurs appropriés. Pour la première fois, cette étude a examiné l'influence de l'élicitation chimique, ultrasonique et thermique de la voie phénylpropanoïde par l'induction de stress à différents stades de germination du haricot commun (*Phaseolus vulgaris*). Avec chaque élicitation, accumulation de marqueurs de stress (H_2O_2, catalase, peroxydase de guaiacol), activités de phénylpropanoïde déclenchant des enzymes (phénylalanine ammoniac-lyase, tyrosine ammoniac-lyase), les composés phénoliques, les capacités antioxydantes et les caractéristiques morphologiques du germe ont été surveillées à différents stades de germination.

Parmi les éliciteurs chimiques, l'acide glutamique 7 mM a provoqué les niveaux les plus élevés de H_2O_2 (0.12 mol/g) à 96 h de temps de germination, tandis que les activités maximales de catalase (23.2 U/mg de protéine) et de peroxydase (3.48 U/mg de protéine) ont été provoqués avec de l'acide glutamique 5 mM comparativement au témoin. L'acide glutamique de 5 mM a plus fortement amélioré les activités de la phénylalanine ammoniac-lyase et tyrosine ammoniac-

lyase, ce qui a entraîné des améliorations de 65% des acides phénoliques totaux et de 2.97 fois les flavonoïdes totaux, et a considérablement amélioré les capacités de DPPH, d'ABTS et d'antioxydants Fe^{2+}. Les élicitateurs chimiques n'ont pas inhibé les caractéristiques morphologiques du germe. Les études par ultrasons ont indiqué les concentrations les plus élevées de H_2O_2 (0.35 mol/g), de catalase (26.04 U/mg de protéines) et de peroxydase de guaiacol (8.80 U/mg de protéines) avec 360 W (60 min) à 96 h de germination. Les marqueurs de stress élevés observés avec les traitements ultrasoniques ont amélioré les activités de phénylalanine ammoniac-lyase et tyrosine ammoniac-lyase, ce qui a entraîné une augmentation des teneurs totales en acides phénoliques, en flavonoïdes et en anthocyanes de 11.7, 6.6 et 11.5 plis, respectivement, par rapport au témoin. En outre, les composés phénoliques induits par les traitements ultrasonores ont considérablement amélioré toutes les capacités antioxydantes testées. De plus, les marqueurs de stress élevés stimulés par un traitement ultrasonique de 360 W (60 min) ont réduit le temps de germination de 60 h, par rapport aux traitements témoins, ainsi que l'indice accru de radicaux, d'hypocotyle et de vigueur des germes de fèves communes.

De plus, les traitements thermiques (25 témoins, 30, 35 et 40 °C) ont considérablement amélioré la biosynthèse des composés phénoliques. Les accumulations les plus élevées de H_2O_2 et de malondialdéhyde ont été obtenues avec 40 °C, tandis que les activités de catalase et de peroxydase de guaiacol ont été le plus souvent provoquées avec 30 °C à 96 h de germination. La phénylalanine ammoniac-lyase a été extrapolée de façon optimale à 30 °C (11 plis de plus que le témoin), tandis que la tyrosine ammoniac-lyase a affiché une augmentation de 97.4%. Ces conditions ont entraîné simultanément une augmentation significative des composés phénoliques et des capacités antioxydantes. Les acides phénoliques totaux ont été multipliés par 7.75, les flavonoïdes totaux par 69.3% et la teneur totale en anthocyanes par 2.29 plis, comparativement

aux témoins. De plus, les marqueurs de stress élevés associés aux traitements à 30 °C étaient non létaux par rapport aux caractéristiques morphologiques des germes, comparativement à d'autres niveaux de température étudiés. Pour les études cinétiques, les taux d'accumulation, k (h^{-1}), estimés pour le H_2O_2 et le malondialdéhyde, ont montré une tendance à la hausse avec l'augmentation de la température. Toutefois, les taux d'activation observés pour les enzymes déclenchant la catalase, la peroxydase de guaiacol et la phénylpropanoïde étaient les plus élevés à 30 °C. De plus, les valeurs d'énergie d'activation (E_a-kJ/mol) estimées pour les acides phénoliques totaux, les flavonoïdes et les anthocyanes étaient de 30.40, 63.98 et 208.63 kJ/mol, respectivement, ce résultat se synchronisant avec les taux d'accumulation élevés, k (h^{-1}) estimé pour les acides phénoliques et les flavonoïdes, par rapport aux anthocyanes.

En conclusion, le traitement ultrasonique de 360 W (60 min) à 96 h de germination a engendré des composés phénoliques ayant une capacité antioxydante maximale, comparativement à d'autres traitements étudiés. Ainsi, l'élicitation contrôlée peut servir de techniques de biotraitement alternatives pour la production d'aliments fonctionnels naturels.

CONTRIBUTION OF AUTHORS

This thesis consists of seven chapters, presented in the form of manuscripts and in accordance with the guidelines for thesis preparation provided by the Faculty of Graduate and Postdoctoral studies. Chapter I consists of a general introduction section with a brief perspective on phenolics concentrations of common bean and how elicitation is a potential method to enhance its concentrations and health benefits. It also details the justification and objectives of the current study. Chapter II provides a comprehensive review of literature on common bean biochemical composition, phenolic distribution among common bean cultivars, phenolic biosynthesis, health benefits, sprouting, elicitation principles and types applied in plant foods. Chapters III, IV, V, and VI constitute the main body of the thesis and have been submitted for publications. Chapter VII comprise of general conclusions, contribution to knowledge and recommendations for future work.

Josephine O. Ampofo reviewed the literature, designed the experiments, conducted all experiments, analyzed data and prepared manuscripts for journal submissions and publications. Professor Michael Ngadi provided funding for Josephine O. Ampofo, guided the design and conduction of experiments, provided laboratory space and supplies for experiments, advice on direction and editing of manuscripts, whereas Professor Hosahalli S. Ramaswamy assisted with study design and manuscript editing. Ebenezer M. Kwofie participated with editing of the nutrient-density of manuscript.

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Manuscripts:

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4CL	4-coumaryl CoA ligase
ABTS	2, 2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) scavenging capacity
AC	Adenylyl cyclase
ANI	Antinutritional index
ANOVA	Analysis of variance
ANTH	Anthocyanins
AOAC	Association of Analytical Communities
BAPNA	Nα-Benzoyl-DL-Arginine-p-Nitroanilide
C4H	Cinnamate-4-hydroxylase
CAM	Calmodulin
cAMP	Cyclic Adenosine monophosphate
CAT	Catalase
CBLs	Calmodulin-like proteins
CDPKs	Calcium-dependent kinases
CE	Catechin equivalent
CHI	Chalcone isomerase
CHR	Chalcone reductase
CHS	Chalcone synthase
CoA	Coenzyme A
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl free radical scavenging capacity
EDTA	Ethylenediamine tetra acetic acid
ET	Ethylene
FRAP	Ferric reducing antioxidant potential assay
GA	Glutamic acid
GABA	Gamma-aminobutyric acid
GAE	Gallic acid equivalent

GPX	Guaiacol peroxidase
GRAS	Generally regarded as safe
H_2O_2	Hydrogen peroxide
IDF	Insoluble dietary fiber
IP3	1,4,5-triphosphate
JA	Jasmonate
KI	Potassium iodide
L-DOPA	Levo dihydroxy phenylalanine
LED	Light emitting diodes
LIM	Score of nutrients to be limited
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
MQRA	Multiple quadratic regression analysis
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
NRF	Nutrient rich food index
ODD	Oxoglutarate-dependent dioxygenase
OH	Hydroxyl free radical antioxidant capacity
ORAC	Oxygen radical absorbance capacity
PAL	Phenylalanine ammonia-lyase
PC1	Principal component 1
PC2	Principal component 2
PCA	Principal component analysis
PKA	Protein kinase A
PKC	Protein kinase C
PKS	Polyketide synthase
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
POD	Peroxidase

PTAL	Bifunctional ammonia-lyase
QRA	Quadratic regression analysis
RE	Rutin equivalent
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRR	Ratio of recommended to restricted nutrients
SA	Salicylic acid
SAIN	Score of nutritional adequacy of individual foods
SDF	Soluble dietary fiber
TAL	Tyrosine ammonia-lyase
TCA	Trichloroacetic acid
TDF	Total dietary fiber
TEAC	Trolox equivalent antioxidant activity assay
TFC	Total flavonoids content
TIA	Trypsin inhibitor activity
TOSC	Total oxyradical scavenging capacity
TPA	Total phenolic acids
TPI	Total polyphenol index
US	Ultrasound
USDA	United States Department of Agriculture

CHAPTER I

1. INTRODUCTION

Pulses such as common beans, lentils, chickpeas, cowpeas, green peas, mung beans, dry peas etc., have contributed to human nutrition over the years. Common beans (*Phaseolus vulgaris*) are well known for their low-fat content and appreciable levels of protein, carbohydrate, minerals, and B-vitamins, with well-known cultivars including kidney, pinto, navy, black, cranberry, pink, small red and great northern beans (Gan et al., 2016; Magalhâes et al., 2017; Sabaté and Soret, 2014). Besides their nutrient-density, research have shown that common beans have additional health benefits due to the presence of secondary metabolites such as phenolics, carotenoids, lectins, and saponin (Deng and Lu, 2017; Xia et al., 2017; Zhao et al., 2016).

In terms of chemical structure, phenolic compounds are secondary metabolites with one or more hydroxyl groups directly attached to a benzene ring, as described by Singh et al. (2015) and Haminiuk et al. (2012). According to Ouchemoukh et al. (2016) over 8,000 phenolic compounds have been identified and isolated in plants, and categorized into groups such as phenolic acids (e.g., hydroxybenzoic and hydroxycinnamic acids), flavonoids (e.g., isoflavonoids, flavonols, flavones etc.,), tannins (hydrolysable and condensed tannins) and stilbenes depending on the number of hydroxyl groups present in their structure, as well as the structural elements linking benzene rings (Singh et al., 2016a). Foods that are rich in phenolics have been shown through epidemiological studies to offer health benefits such as antioxidant, anti-inflammatory, antidiabetic, anticancer, antiobesity, antimutagenic and antimicrobial properties (Curran, 2012; Frassinetti et al., 2015; Gan et al., 2016; García-Lafuente et al., 2014).

However, depending on factors such as cultivar, growing conditions, growth stage, geographical location, storage and processing conditions, phenolic levels vary among various common bean species. For instance, total flavonoids contents of green, black turtle, and pinto beans cultivars were reported as 0.50, 70.2 and 2.99 mg CE/g, respectively (Jiratanan and Liu, 2004; Tan et al., 2017; Xu et al., 2007). Also, total phenolic acids levels for red gram, dry and dark red kidney bean cultivars were reported as 3.16, 5.55 and 60.80 mg GAE/g, respectively (Khandelwal et al., 2010; Nergiz and Gokgoz, 2007; Sutivisedsak et al., 2010). Taking advantage of the health benefits of phenolic-dense foods, and the wide consumption of common beans, harnessing mechanisms that will increase phenolic accumulation in common beans based on their principle of biosynthesis will help improve their phenolic value, especially in cultivars with limited concentrations. According to Randhir et al. (2009) phenolics in plants are produced mainly through the phenylpropanoid pathway, although other metabolic pathways such as the pentose phosphate and shikimate pathways also contribute to their biosynthesis. Phenolic compounds are secondary metabolites produced in plants in response to environmental stress and as signaling compounds involved with various physiological processes (e.g., genetic expressions, pollination etc.). Thus, developing mechanisms that can induce non-lethal stress levels in plant foods at different growth stages can alter physiological processes and trigger phenolic biosynthesizing pathways at increased rates for higher phenolic yield.

Sprouting has been documented in literature as a growth stage involving complex metabolic processes, such as the hydrolytic breakdown of stored macromolecules into simpler forms for embryonic growth, as well as the novo-synthesis of non-existing compounds (Shi et al., 2010; Wang et al., 2005). Furthermore, several works on sprouting of common beans has been reported to increase contents of phenolics depending on cultivar and sprouting conditions

(e.g., time, temperature, illumination etc.,), with this observation attributed to the activities of enzymes responsible for triggering the phenylpropanoid pathway along the sprouting process (Aguilera et al., 2014; Wu et al., 2012; Lopez et al., 2013). However, sprouting is a slow process and requires the application of the appropriate technology to enhance its yield of phenolics. This can be achieved through two main key mechanisms including: (a) seed genetic manipulation and (b) modification of physiological processes. Due to the controversy related to the production and negative effects of genetically modified foods, modification of plant secondary metabolites such as phenolics through physiological modification mechanisms seems to be the potential alternative (Gawlik-Dziki et al., 2012). According to Owolabi et al. (2018), physiological modification of phenolics biosynthesis can be achieved by the scientific principle of elicitation. Elicitation involves the induction of stress in a biological system, leading to changes in its physiological and morphological properties through the application of an elicitor (Liu et al., 2019). During plant elicitations, stress induced along the process enhance accumulation of phenolics at higher levels, due to their higher demand as protective compounds against effects of the applied elicitor.

1.1 Thesis Research Hypothesis

Controlled elicitations of plants at their sprouting phase of development can maximize accumulation of their phenolics compounds. Although literature has documented vast data on enhanced phenolic yield with elicitation, existing data suggest their wide applications in bioreactor systems, with extremely scanty applications in plant foods such as edible common bean sprouts (Swieca et al., 2014). Notwithstanding, authors such as Swieca (2016), Yang et al.

(2015) and Limon et al. (2014) observed increased contents of phenolic compounds for elicited sprouts of lentils, soy and kidney beans, compared to their untreated forms.

However, with the limited data on elicited sprouts, mechanisms by which elicitation induce stress and stimulate activities of phenolic biosynthesizing enzymes for maximization of phenolic compounds during sprouting is still not existing.

Based on the aforementioned literature gaps, the hypothesis of this thesis was that, controlled elicitation of common bean sprouts can improve accumulation and antioxidative capacities of their phenolic compounds, through the principle of stress induction and stimulations of phenylpropanoid triggering enzymes. Results of this study will be significant in aiding the design of processing systems towards the production of phenolic-rich common bean sprouts. Furthermore, applications of elicitation can provide an easy, cheap, sustainable and green approach to diversify nutraceutical-value of the common beans market.

1.2 Thesis Research Objectives

The main objective of the current thesis was to investigate how different elicitations influence phenolic biosynthesis and antioxidant capacities of common beans along the process of sprouting. Special emphasis was placed on influence of elicitation on triggering of the phenylpropanoid pathway. Specific objectives of this study have been set to the following:

i) Study impact of sprouting on nutrient-density and antioxidant capacities of six common bean (*Phaseolus vulgaris*) cultivars.

ii) Evaluate effect of different levels of chemical (glutamic acid and NaCl) and physical (ultrasound and temperature) elicitations on stress accumulation and stimulation of phenolic

triggering enzymes involved with the phenylpropanoid pathway at different sprouting stages of common beans.

iii) Correlate information on stress induction and activities of phenylpropanoid triggering enzymes with accumulation of phenolic compounds, antioxidative and morphological properties of common beans at different sprouting stages.

iv) Correlate thermal elicitation studies with kinetic modelling of phenolic compounds during treatments.

CHAPTER II

LITERATURE REVIEW

2.1 Common bean (*Phaseolus vulgaris*)

Common bean are staple foods of nutritional and functional value. According to Broughton et al. (2003) the name common bean can be attributed to its long history of cultivation as the main grain legume for human consumption. Botanically, common bean belongs to the family Fabaceae and genus *Phaseolus,* with over 14,000 cultivars (Sathe, 2002). Their seeds can be cooked as a vegetable or added to soups and sauces. They can also be dried and milled into flour for use in diverse food systems such as baked goods, extruded products, soups, sauces and porridges (Rizzello et al., 2014).

2.2 Major biochemical composition

2.2.1 Carbohydrates

Carbohydrates constitute the major component of common bean. Of the dry matter, carbohydrate account up to 50-70 % depending on the cultivar, with starch and non-starch (e.g., cellulose, pectin etc.,) polysaccharides making up major components (Hoover et al., 2010; Bouchenak and Lamri-Senhadji, 2013).

2.2.2 Proteins

Common beans are excellent dietary source of protein, ranging between 15-31 g/100 g based on the cultivar. Proteins are composed of amino acid units consisting of a central carbon atom bound to an amino group, carboxyl group, hydrogen atom, and a side chain. Beans proteins

are divided into fractions including, globulins, albumins, prolamin and glutelin (Adebowale et al., 2007). Literature has shown common bean proteins to have low digestibility. According to Yu (2005) the low digestibility, of common bean proteins can be attributed to the β-sheet structure and glycosylation of phaseolin and legumin, as well as disulfide bonds of albumin and glutelin which impart a compact rigid hydrophobic structure, less accessible for proteolysis and final digestion.

2.2.3 Minerals and vitamins

Common beans are essential sources of food minerals such as iron, zinc, copper, phosphorous and aluminum (Shimelis and Rakshit, 2005). Zinc, copper, phosphorus, and aluminum levels in common bean cultivars are in the ranges of 10.1-109, 2.8-10.9, 15.8-64.6, and 6.7-14.4 μg/g respectively (Wu et al., 2005). Also, common beans are rich in B-complex vitamins, with thiamine, riboflavin, niacin, pyridoxamine and folate contents ranging between 0.81-1.32, 0.112-0.411, 0.85-3.21, 0.299-0.659 and 0.148-0.676 mg/100 g, respectively (Augustin et al., 2000).

2.2.4 Antinutritional compounds

Common beans comprise of antinutrients that limit digestion and absorption of their nutritional molecules. Well known examples of antinutrients found in common beans include tannin, phytate, enzyme inhibitors, lectins and oligosaccharides etc. (Tadele et al., 2015).

Depending on the cultivar, tannin levels in common beans vary between 0.00-0.93 %. They are divided into condensed and hydrolysable tannins. Condensed tannins have been shown to exert an effect of decreasing digestibility, whereas hydrolysable tannins may cause toxic

digestive tract effects (Akande et al., 2010). However, lower levels of tannins are reported to offer health benefits. For instance, Bawadi et al. (2005) reported that tannins isolated from black beans inhibited growth of Caco-2 colon, MCF-7 and Hs578T breast and DU 145 prostatic cancer cells.

Phytate is regarded as the primary storage form of phosphate and inositol. Due to the presence of negatively charged phosphate groups in phytate, they are able to form complexes with mineral ions (e.g., Zn^{2+}, $Fe^{2+/3+}$, Mn^{2+}), thus limiting their intestinal absorption (Walter et al., 2002). However, according to Muller (2001) they are reported to serve as antioxidants due to the anti pro-oxidant metal ion chelating ability of their phosphate groups.

Enzyme inhibitors found in common beans include protease and amylase inhibitors. Protease inhibitors in beans are divided into the Bowman-Birk and Kunitz types. These are low molecular weight proteins that form complexes with proteases, leading to reductions in protein digestibility, quality, amino acid availability as well as leading to pancreatic hypertrophy (Bharathi and Arun, 2012). However, at reduced levels they are reported to help inhibit the proliferation of certain cancerous cells (Finotti et al., 2006). Furthermore, amylase inhibitors form complexes with amylases, reducing digestion of starch and glycogen (Bharathi and Arun, 2012).

Lectin content in common beans ranges between 2.4-5 %, depending on cultivar. Lectins bind to specific carbohydrate receptors on erythrocytes of the intestinal mucosal membrane, causing epithelial lesions and reduced absorption of digestive metabolites. However, threshold levels of lectin have been reported to exert positive health benefits. For instance, Shi et al. (2007) reported on extracted red kidney bean lectins that showed *in vitro* inhibition of HIV-1 RT.

Oligosaccharides such as raffinose, stachyose and verbascose are responsible for the flatulence, stomach discomfort, bloating and abdominal cramps associated with common bean consumption. These negative influences are due to the absence of α-galactosidase in the small intestine necessary for hydrolysis of α-1, 6 linkages in the structure of these oligosaccharide groups (Bharathi and Arun, 2012). Despite their undesirable effects, oligosaccharides have been linked to helping reduce risks of intestinal cancer, obesity and cardiovascular diseases (Onder and Kahraman, 2009).

2.2.5 Phenolic compounds

Common beans possess a wide array of phenolic compounds mainly distributed in the seed coat, with flavonoids and phenolic acids being the principal components (Amarowicz and Pegg, 2008). Lin et al. (2008) investigated the phenolic profile of different cultivars of pinto and red kidney beans, reporting the predominant phenolics as glucosides of kaempferol and quercetin. Also, Dinelli et al. (2006) investigated flavonoid profile of Italian beans and reported ranges of 0.19-0.84 g/kg, with kaempferol monoglucoside, kaempferol 3-O-glucoside, and kaempferol 3-O-xylosylglucoside being the most dominant.

2.3 Phenolic compounds in common beans

Phenolic compounds are diverse groups of secondary metabolites, with literature documenting about 10,000 different types of phenolic structures (McCallum, 2010; Saltveit, 2010). Phenolics share a common structure of carboxylic acid function (C6-C1 skeleton) with at least one phenol unit and one or more hydroxyl substituents (Gan et al., 2017; Akyol et al., 2016). They give common bean seeds their diverse colors and are widely distributed in the seed

coat although some concentrations also occur in the cotyledon (Aquino-Bolaños et al., 2016). Literature has documented wide data on phenolic profile of common beans, with concentrations differing based on cultivar, geographical location, growth conditions and extraction methods. Broadly, phenolics are divided into three groups namely phenolic acids, flavonoids and tannins as discussed in the following section.

2.3.1 Phenolic acids

Phenolic acids are aromatic compounds characterized with a benzene ring linked with one or more hydroxyl or methoxy groups. Biosynthesis of phenolic acids begins with the shikimate pathway, where shikimic acid acts as substrate for the production of gallic acid and phenylalanine. Phenylalanine then serves as a precursor for the phenylpropanoid pathway, where phenylalanine is acted upon by phenylalanine ammonia-lyase (PAL) into cinnamate which serves as an intermediate for the production of subsequent phenolics (i.e., *p*-coumarate, and 4-coumarolyl-COA) (Jendresen et al., 2015; Le Roy et al., 2016; Liu et al., 2015). Phenolic acids are classified into two main groups, namely hydroxybenzoic and hydroxycinnamic groups as discussed below:

2.3.1.1 Hydroxybenzoic acids

Hydroxybenzoic acids have a C6-C1 structure (Fig. 2.1), and normally occur in foods as glycosides.

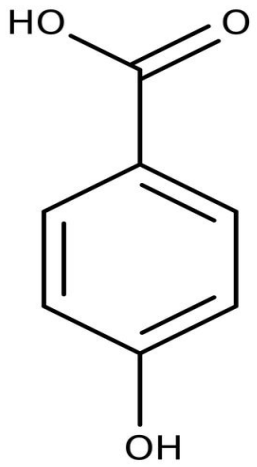


Figure 2.1: Basic structure of a hydroxybenzoic acid (Singh et al., 2017)

Twelve different types of hydroxybenzoics have been identified in common beans, with gallic, p-hydroxybenzoic, protocatechuic, vanillic, and syringic acids being the most prevalent. An early study by Aguilera et al. (2011) reported total hydroxybenzoics levels of 21.93 and 84.92 μg/g, in uncolored cannellini and colored pinto beans, respectively. From this study, hydroxybenzoics profiling of uncolored cannellini bean comprised of *p*-hydroxybenzoic (4.30 μg/g), *p*-hydroxyphenyl acetic (6.92 μg/g) and vanillic (10.71 μg/g) acids, whereas colored pinto bean was made up of protocatechuic (2.40 μg/g), *p*-hydroxyphenyl acetic (8.42 μg/g), *p*-hydroxybenzoic (12.20 μg/g), vanillic (17.01 μg/g) and salicylic (44.89 μg/g) acids. Another study by Chen et al. (2015) observed *p*-hydroxybenzoic acid ranges of 29.08-71.26 μg/g, as the key hydroxybenzoic present in darkening cultivars of cranberry bean. Furthermore, soluble and

insoluble dietary fiber fractions of dark bean were reported by Dueñas et al. (2016) to consist of total hydroxybenzoics levels of 7.3 and 20.4 μg/g, respectively.

2.3.1.2 Hydroxycinnamic acids

Hydroxycinnamic acids have a C6-C3 structure, as shown in Figure 2.2.

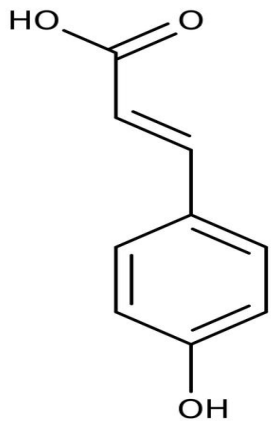


Figure 2.2: Basic structure of a hydroxycinnamic acid (Singh et al., 2017)

They mostly occur in foods as esters with sugars or quinic acids. Sixteen hydroxycinnamics have been identified in common bean, with caffeic, *p*-coumaric, sinapic and ferulic acids being the most abundant. According to Duenas et al. (2015) hydroxycinnamics account for about 50% of the total phenolic content in kidney beans. In another study by García-Lafuente et al. (2014), phenolic profiling of white kidney and round purple beans revealed aldaric derivatives of ferulic, *p*-coumaric and sinapic acids as the main hydroxycinnamics. Also, Luthria and Pastor-Corrales (2006) evaluated hydroxycinnamics of 15 common bean cultivars and detailed mean values of *p*-coumaric, sinapic and ferulic acids to be 6.3, 7.0 and 17.8 mg/100 g, respectively.

2.3.2 Flavonoids

These are the most abundant secondary metabolites and are mainly responsible for the different coloration and flavor of common bean. Flavonoids are localized in cell vacuoles of plant tissues as conjugates in glycosylated or esterified forms, but infrequently occur as aglycones (Nayak et al., 2015). Flavonoids are composed of two aromatic rings (A and B) joined by a 3-carbon-bridge (C6-C3-C6 structure). Their A-ring is obtained from the acetate pathway, whereas the B-ring is obtained from the shikimic acid pathway. According to Alminger et al. (2014) polarity of flavonoids is determined by the presence of a carbonyl group at C4 position, followed by hydroxyl groups at positions C3 and the presence of sugar moieties. Literature has documented vast number of individual flavonoids, and are categorized into six main groups including isoflavonoids, flavonols, flavones, flavanones, flavanols and anthocyanins (Amarowicz and Pegg, 2008).

Table 2.1: Total phenolic acids and flavonoid contents of some reported common bean varieties

Cultivar	Phenolic type	Reference
	Total phenolic acids	
Green bean	0.80 mg GAE/g	Jiratanan and Liu, 2004
Kidney bean	0.46-3.90 mg GAE/g	Aguilera et al., 2014
Pinto bean	3.74 mg GAE/g	Akillioglu and Karakaya, 2010
Black bean	1.58-4.38 mg GAE/g	Treviño-Mejia et al., 2016
Broad bean	23.9 mg CE/g	Amarowicz et al., 2004
Red bean	1.8 mg GAE/g	Telles et al., 2017
White bean	0.19 mg GAE/g	
	Total flavonoids	
Kidney bean (26 cultivars)	0.19-7.05 mg RE/g	Kan et al., 2016
Black turtle bean	70.2 mg CE/g	Tan et al., 2017
Black bean cultivars	2.51-3.20 mg CE/g	Xu et al., 2007
Pinto bean	2.99 mg CE/g	
Navy bean	0.92 mg CE/g	
Green bean	0.52-6.0 mg CE/g	Jiratanan and Liu, 2004
Dry bean cultivars	0.24-1.43 mg CE/g	Heimler et al., 2005

GAE = gallic acid equivalent, RE = rutin equivalent, CE = catechin equivalent

2.3.2.1 Flavanols and isoflavonoids

These flavonoid groups are primarily found in common bean cultivars with colored seed coats. The most abundant types reported in common beans include catechin, epicatechin, epigallocatechin and epicatechin gallate (Yang et al., 2018). A recent study revealed catechin and proanthocyanidins levels of darkened cranberry beans to range between 142.26-203 and 15.30-35.29 μg/g, respectively (Chen et al., 2015). A similar profiling of 15 common bean cultivars by Mojica et al. (2015) identified 1.75-5.42 and 3.80-12.48 % concentrations of catechin and epicatechin, respectively. Isoflavonoids are groups of flavonoids that are predominantly found in legumes and differ from other flavonoid groups by their B-ring located at the C3 position. The principal types of isoflavonoids distributed in common bean include genistein, daidzein, glycitein, formononetin and their derivatives (Yang et al., 2018).

2.3.2.2 Flavones and flavonols

According to the review of Yang et al. (2018) two groups of flavones (i.e., apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside) and twenty-two groups of flavonols (especially kaempferol, quercetin, myricetin and their derivatives) have been identified in common beans. Baginsky et al. (2013) identified quercetin glucoside (5.1-25.9 mg/kg), quercetin rutinoside (7.4-56.9 mg/kg), quercetin galactoside (8.3-92.4 mg/kg), apigenin galactoside (8.9-168 mg/kg), myricetin glucoside (9.9-41.9 mg/kg) and myricetin (18.8-119.5 mg/3 kg) in 10 faba bean cultivars. Also, Magalhães et al. (2017) identified 1.06 μg/g content of kaempferol dihexoside-rhamnoside in dark bean.

2.3.2.3 Flavanones

Nine groups of flavanones have been discovered in common beans with naringenin and hesperetin glucosides being the most prevalent (Yang et al., 2018). A previous study by Aguilera et al. (2011) detailed 1.29, 3.83 and 4.44 µg/g contents of naringenin derivatives, eriodictyol and sakuranetin, respectively, in seeds of pinto bean. With this same study, 1.20, 1.28, 1.59 and 2.01 µg/g levels of eriodictyol, pinocembrin derivative, sakuranetin and eriodictyol derivative were found in cannellini bean seeds, respectively. Similarly, García-Lafuente et al. (2014) observed 0.14 and 11.30 µg/g levels of hesperitin and naringenin, respectively, in white kidney bean.

2.3.2.4 Anthocyanins

Anthocyanins are made up of anthocyanidins which consist of an aromatic ring A bonded to a heterocyclic ring C (containing oxygen), forming a carbon-carbon bond with a third aromatic ring B. When anthocyanidins are bonded to a sugar moiety, they are referred to as anthocyanins (Singh et al., 2017). Twenty anthocyanins have been discovered in common bean, the most predominant of which include glycosides of cyanidin, malvidin, delphinidin and petunidin, as reported by Lopéz et al. (2013). These authors recognized pelargonidin 3-*O*-glucoside (50.72 µg/g) and cyanidin 3-*O*-glucoside (88.44 µg/g) as the key anthocyanin groups present in dark bean seeds.

García-Lafuente et al. (2014) also reported malvidin, pelargonidin and cyanidin glucosides as the principal anthocyanins present in seeds of round purple bean. Furthermore, Dueñas et al. (2015) observed malvidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside and pelargonidin 3-*O*-glucoside as the central anthocyanins in kidney bean at levels of 0.06, 0.31 and 1.90 µg/g, respectively. Another anthocyanin profiling of 26 kidney bean cultivars by Kan et al. (2016)

demonstrated malvidin, petunidin, pelargonidin, cyanidin and delphinidin ranges of 0-0.27, 0-0.41, 0-0.71, 0-1.44 and 0-4.45 mg/g, respectively, as the prevailing groups.

2.4 Biosynthesis of phenolic compounds

Phenolics are a large group of phenylpropanoid secondary metabolites, synthesized from phenylalanine or tyrosine through a chain of enzymatic reactions. According to Deng and Lu (2017), phenylpropanoids are produced in plants as defense compounds in response to environmental stress, as well as for seed coloring and the formation of pollinating organs. Phenylpropanoids are mainly divided into five groups, including flavonoids, phenolic acids, monolignols, stilbenes and coumarins (Liu et al., 2015; Vogt, 2010). Among these phenylpropanoids, the most widely distributed in common beans are flavonoids, phenolic acids and monolignols, although stilbenes and coumarins are also present but at limited concentrations (Kiselev et al., 2016; Munakata et al., 2014). At the moment, different enzymes (such as ligases, lyases, transferases, oxygenases, and reductases) have been identified and linked to the phenylpropanoid pathway, as reviewed by Deng and Lu (2017). According to the reports of Hou et al. (2013) and Tohge et al. (2012) enzymes associated with the phenylpropanoid pathway are encoded by gene super-families such as cytochrome membrane-bound monooxygenase (P450) gene family, 2-oxoglutarate-dependent dioxygenase (2-ODD) gene family, NADPH-dependent reductase gene family and type III polyketide synthase (PKS III) gene family. In addition to genetic influence, activities of transcription factors (including *v-myb* myeloblastosis viral oncogene homolog, basic helix-loop-helix protein, WD-repeat protein, NAC and WRKY) and mRNAs (including *miR828*) have been associated with the regulation of the phenylpropanoid biosynthesis (Xu et al., 2015).

2.4.1 The general phenylpropanoid pathway

The first steps of the phenylpropanoid pathway are broadly categorized as the general phenylpropanoid pathway. The first enzyme involved with triggering the phenylpropanoid pathway is phenylalanine ammonia-lyase (PAL), which catalyzes the deamination of L-phenylalanine to trans-cinnamic acid (trans-cinnamate) and ammonia. Tran-cinnamate is further reduced to *p*-coumaric acid (4-coumarate) by the action of an NADPH-dependent enzyme, cinnamate-4-hydroxylase (C4H), as shown in Figure 2.3. However, in some plant species tyrosine can directly be converted to *p*-coumaric acid by the action of the enzymes tyrosine ammonia-lyase (TAL) or bifunctional ammonia-lyase (PTAL), thus bypassing the trans-cinnamic and C4H intermediates (Barros et al., 2016; Jendresen at al., 2015).

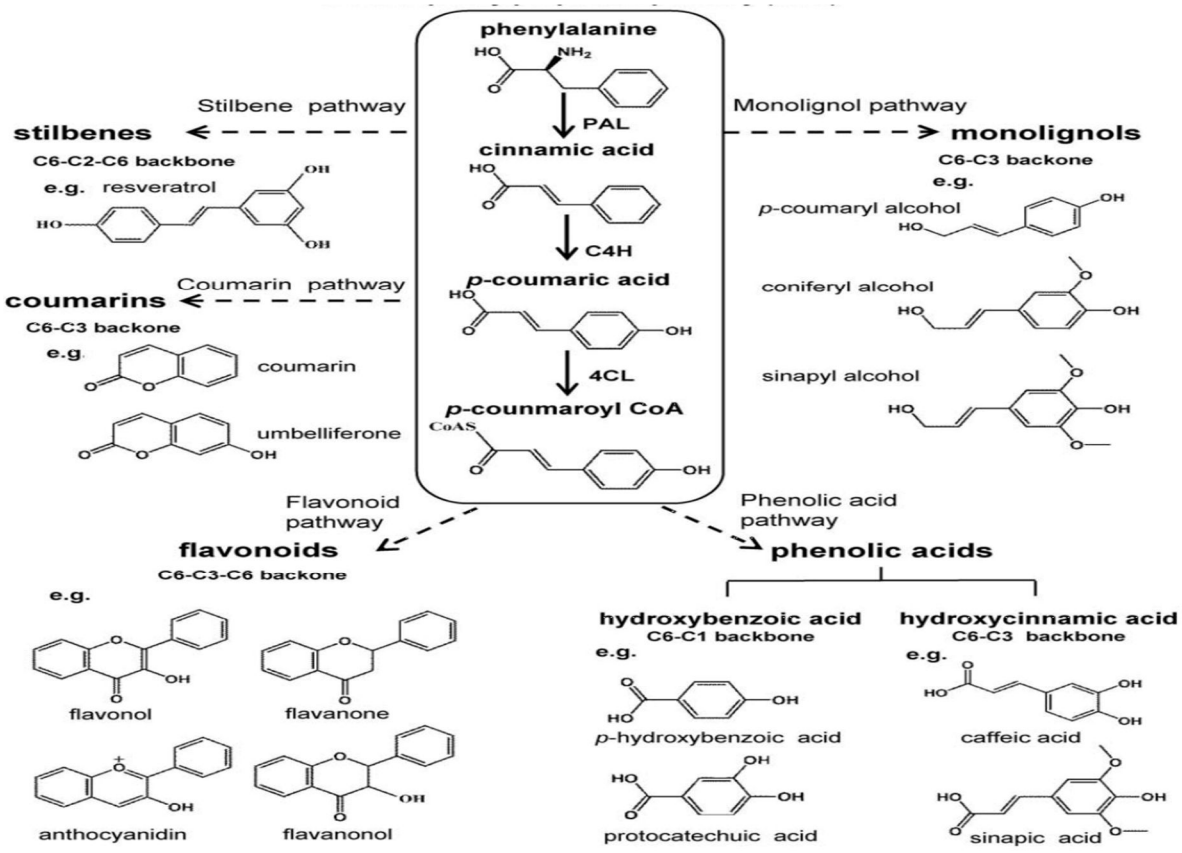


Figure 2.3: Phenylpropanoid pathway for biosynthesis of phenolics. Abbreviations: PALphenylalanine ammonia-lyase; C4H- Cinnamate 4-hydroxylase; 4CL- 4-coumaryl CoA ligase. (Deng and Liu, 2017).

Afterwards, the enzyme 4-coumaryl CoA ligase (4CL) then catalyzes the conversion of *p*-coumaric acid to *p*-coumaroyl-CoA, which serves as the carbon source for other branched pathways leading to the formation of phenolic acids, flavonoids and lignin (Vogt, 2010).

For flavonoid synthesis, one molecule of p-coumaroyl-CoA is condensed with three malonyl-CoA molecules by the action of the enzyme chalcone synthase (CHS) into naringenin chalcone (2′,4′,6′, 4 -tetrahydroxychalcone) (Jez and Noel, 2000; Falcone-Ferreyra et al., 2012). Additionally, a second enzyme which has been identified only in legumes as chalcone reductase (CHR), has also been reported to work in synergy with chalcone synthase. Chalcone reductase acts on intermediate metabolites produced by chalcone synthase and redirects them to the biosynthesis of isoliquiritigenin chalcone (4, 2′,4′-trihydroxychalcone) (Bomati et al., 2005), thus, explaining why isoliquiritigenin chalcone is mainly found in legumes.

Following this, the next enzymatic step is induced by chalcone isomerase (CHI) by catalyzing the stereo specific cyclization of either naringenin or isoliquiritigenin chalcone to flavanone (i.e., naringenin flavanone or isoliquiritigenin flavanone). Furthermore, although naringenin flavanone becomes the intermediate for biosynthesis of other flavonoid subgroups (e.g., flavones, flavanonols, flavonols, anthocyanins, and isoflavones), isoliquiritigenin flavanone can only be used as an intermediate for legume-specific isoflavonoids such as genistein, daidzein and glycitein etc., (Ngaki et al., 2012).

2.5 Factors affecting composition and concentration of phenolic compounds

Besides the influence of biosynthetic enzymes, concentration and composition of phenolics in common bean are also influenced by other factors such as environmental conditions, cultivar, developmental stage, storage and processing conditions.

2.5.1 Genetics and environmental conditions

Cultivar, geographical distribution and environmental conditions under which common beans are grown immensely contribute to their variations in phenolic levels and composition. Over the past years, researchers have investigated effects of these factors on phenolics in common beans, with their results linking significant evidence to these effects. For instance, Islam et al. (2003) investigated the effects of genetic pool on total phenolic profile of common beans from Middle and North Andean America. From their study, common beans from the Middle American gene pool reported the greatest levels of total phenolic content, compared to the North Andean gene pool. Another study by Espinosa-Alonso et al. (2006) evaluated the effect of cultivar and geographical location on total phenolic content of common beans, concluding that cultivars have a greater effect on phenolic accumulation in common beans than geographical location.

Similarly, Barampama and Simard (1993) observed that proanthocyanidins accumulation in common beans was significantly influenced by both cultivar and growth environmental conditions. Nevertheless, the work of de Mejia et al. (2003) concluded that proanthocyanidins levels in common beans are mainly influenced by cultivar type, compared to growth environmental conditions.

2.5.2 Sprouting

Sprouting is a simple, inexpensive and sustainable technology to increase bioactive compounds such as phenolics in seeds (Shohag et al., 2012). Sterilization and soaking of seeds precede the process of sprouting. Sprouting begins with the imbibition of water and ends with the emergence of radicle from the seed coat. Factors such as sprouting time, temperature, pH, soaking, light, humidity, and oxygen are the conditions that determine the accumulation of

metabolites during sprouting (López et al., 2013). However, because morphological features and enzymatic processes changes with time, the purpose of sprouting should be clearly defined before commencement. For edible beans, sprouting stage of 3-5 days has been reported to be enough for bean sprouts to attain an edible length (Gan et al., 2017). During sprouting, sprouts are watered intermittently to keep them hydrated and also prevent microbial growth.

2.5.2.1 Mechanism of sprouting and accumulation of phenolic compounds

From the review report of Gan et al. (2017) sprouting of edible seeds does not only lead to the breakdown of complex macromolecules (i.e., carbohydrates, fats and proteins) into simple soluble forms (i.e., simple sugars, free amino acids, fatty acids and organic acids), but also leads to increased accumulation of secondary metabolites such as phenolics.

Accumulation of phenolics during sprouting has been attributed to two main factors including activities of enzymes involved with the phenylpropanoid pathway and activities of cell wall hydrolysing enzymes to release bound phenolics (Agati et al.., 2012) conjugated with complex carbohydrate molecules, depending on the sprouting conditions. Xue et al. (2016) observed a 1.54-fold significant increment of total phenolic content in 6-day sprouted black beans, compared to their untreated forms. This study also reported 34.12% significant increase in total flavonoids, compared to their unsprouted forms.

However, some studies have also reported reduced levels of phenolics as a result of sprouting. For instance, Guajardo-Flores et al. (2013) detailed reduced flavonoid levels (0.50 mg GAE/g DW) with 5-day sprouted black bean, compared to its control (0.90 mg GAE/g DW). Variations in these observations may be attributed to several factors such as the activities of polyphenol oxidase, peroxidases, method of calculation, and experimental design of the

sprouting process. Polyphenol oxidase and peroxidases are known to catalyze the mobilization of simple phenolics into formation of quinones (responsible for sprout browning) and monolignols (for lignification), respectively. Additionally, calculations based on only cotyledon analysis will report lower phenolic levels, than calculations including the whole sprouted bean (cotyledon and seed coat), as observed with the works of Guajardo-Flores et al. (2013) and Xue et al. (2016), respectively.

Table 2.2: Total phenolic contents of common bean seeds and sprouts

Cultivar	Total phenolic content		Reference
	Raw seed	*Sprout*	
Kidney bean (*Phaseolus vulgaris*)	370 mg GAE/100 g	110-420 mg GAE/100 g	Aguilera et al., 2014
Kidney bean (*Phaseolus vulgaris*)	34 mg GAE/100 g	40-60 mg GAE/100 g	Wu et al., 2012
Pinta bean (*Phaseolus vulgaris*)	30 mg GAE/100 g	15-45 mg GAE/100 g	Aguilera et al., 2014
Mung bean (*Vigna radiata*)	0.40 mg GAE/g	3.4 mg GAE/g	Pajak et al., 2014
Soybean (*Glycine max*)	0.1 mg GAE/g	0.41-1.20 mg GAE/g	Huang et al., 2014
Sword bean (*Canavalia gladiata*)	40 mg GAE/100 g	30-58 mg GAE/100 g	Wu et al., 2012
Adzuki bean (*Vigna angulariz*)	43 mg GAE/100 g	35-80 mg GAE/100 g	Wu et al., 2012
Black mung bean (*Vigna radiata*)	180 mg GAE/100 g	140-902 mg GAE/100 g	Gan et al., 2016

TPC = Total phenolic content, GAE = gallic acid equivalent, CE = catechin equivalent

2.6 Antioxidant capacity of common beans phenolic compounds

Literature has detailed vast data on health benefits of consuming phenolic-rich foods such as antidiabetic, antithrombotic, antimutagenic, anticancer, antitumor and antimicrobial effects (Mendoza-Sanchez et al., 2016). However, it should be highlighted that, these health benefits are related to the antioxidant capacity of phenolics. Antioxidant capacity of a phenolic compound is dependent on the degree of glycosylation, as well as the number and position of hydroxyl groups in relation to the carboxyl functional group present in its chemical structure (Singh et al., 2017). Phenolics act as antioxidants by scavenging free radicals and chelating metal ions. Free radicals and metal ions have negative effects on biological systems by stealing electrons from healthy cells and causing them to be unstable. In order to be stable, these unhealthy cells further take electrons from neighbouring cells, resulting in increased levels of harmful reactive oxygen species (ROS) and final oxidative damage as the cycle continues (Gill and Tuteja, 2010). Thus, the need for phenolics as antioxidants to protect against oxidative stress. Phenolic compounds act as antioxidants by scavenging free radicals, decomposing oxidation products and chelating metal ions through their hydrogen and electron donating capacities (Balasundram et al., 2006), causing these oxidative radicals to become stable.

The antioxidant capacity of phenolics has been determined by various *in vitro* mechanisms such as ferric reducing antioxidant potential assay (FRAP), oxygen radical absorbance capacity (ORAC), hydroxyl free radical scavenging assay (OH), 1, 1-diphenyl-2-picrylhydrazyl free radical scavenging assay (DPPH), Trolox equivalent antioxidant activity assay (TEAC), 2, 2′-azino-bis (3- ethylbenzothiazoline-6-sulfonic acid) free radical scavenging assay (ABTS) and total oxyradical scavenging capacity (TOSC) (Ombra et al., 2016). Xu et al. (2007) evaluated antioxidant capacity of different common bean cultivars, with their reported

ORAC scavenging values ranging within 3.30-92.73 μmol TE/g, whereas Wang et al. (2016) observed antioxidant capacity of phenolic extracts from black turtle bean to be 49.2 μmol TE/g. The antioxidant capacity of phenolic extracts from different cultivars of cranberry beans was reported to range within 2.11-287.87 μmol TE/g (Chen et al., 2015).

Also, antioxidant capacity of phenolic extracts from seed coat of 26 white and black kidney bean cultivars were reported by Kan et al. (2016) to range between 4.00 and 491 μmol TE/g, respectively. Similarly, phenolic extracts from red kidney, pinto, black kidney, and small red beans were reported to possess antioxidant capacities of 516, 567, 602 and 622 U/g, respectively (Zhao et al., 2014). Furthermore, Orak et al. (2016) reported ABTS and FRAP scavenging values of phenolic extracts obtained from different cultivars of Turkish white bean to be within ranges of 3.50-5.17 μmol TE/g and 7.99-11.2 μmol Fe^{2+}/g, respectively. In another work, the ABTS values of black and small red bean phenolic extracts were reported as 170 and 211 μmol TE/g, respectively, whereas their ORAC values were observed to be 1241 and 1079 μmol TE/g, respectively (Sancho et al., 2015). Similarly, another research evaluating antioxidant potential of phenolic extracts from different cultivars of Sarconi beans, detailed DPPH scavenging values within ranges of 2.78-16.9 EC_{50} (mg/g) (Romani et al., 2013).

In addition to *in vitro* antioxidant capacity of phenolics from common beans, *in vivo* studies involving animals have also confirmed antioxidant potential of phenolics extracted from common beans. For instance, Xu and Chang (2011) reported that phenolic extracts from pinto and black beans exhibited significant intracellular antioxidant capacities in a concentration dependent manner. Thus, from all these reports it can be concluded that there is a positive correlation between consumption of common beans and protection from negative oxidative stress, as a result of its phenolic composition.

2.7 Elicitation of secondary metabolites in plant foods

Based on the principle that phenolics are produced as intermediate compounds from the interaction between a plant and its environment, elicitation has been reported as a tool to manipulate plant physiological processes towards enhanced phenolic biosynthesis (Owolabi et al., 2018). Recent studies have shown that, elicitation of plant foods at different growth stages (e.g., sprouting, seedling, pre-harvesting and post-harvesting stages) can help improve accumulation of secondary metabolites such as phenolics (Wei et al., 2011; Sritongtae et al., 2017; Ding et al., 2018; Hao et al., 2016).

2.7.1 Principle of elicitation

Basically, elicitation is the mechanism of inducing biosynthesis of plant secondary metabolites, in order to enhance viability, survival and competitiveness of the plant in the face of environmental stress, via manipulation of its physiological and morphological processes (Patel and Krishnamurthy, 2013; Radman et al., 2003). Elements that are used to induce these physiological and morphological changes are known as elicitors/ stressors (Baenas et al., 2014b; Zhao et al., 2005). Depending on their origin and molecular weight, elicitors are categorized into two main types, namely biotic and abiotic elicitors.

As reviewed by Liu et al. (2019), biotic elicitors are the types that are obtained from biological origin, either from the plant itself or from pathogens. Notable examples of biotic elicitors include polysaccharides, phytohormones, oligosaccharides, proteins, fatty acids, and diverse biological extracts (Vasconsuelo and Boland, 2007; Natella et al., 2016; Lan et al., 2016). Contrary to biotic forms, abiotic elicitors are derived from chemical or physical sources. Examples of chemical sources include diverse salts (such as NaCl, Na_2SeO_3, Na_2SeO_4, $ZnSO_4$

etc.,) whereas physical source of abiotic elicitors include temperature, hypoxia, ultrasound, light, electrolyzed water, plasma treatment and high pressure techniques (Vale et al., 2015; Ding et al., 2018; Yang et al., 2013; Hao et al., 2016; Puligundla et al., 2017).

During elicitation, accumulation of secondary metabolites is achieved through a series of transduction process (Fig. 2), depending on the receptor (plant) and elicitor nature. The first step of elicitation is known as signal perception, where receptors located on the cell wall, plasma membrane or cytosol of the plant recognize the applied elicitor. Following signal perception, there is the induction of signal transductions where numerous second messengers such as phosphorylation and dephosphorylation of plasma membrane and cytosolic proteins, influx of Ca, production of reactive oxygen and nitrogen species (ROS, RNS), NADPH activation, extracellular alkalinisation, cytoplasmic acidification, Cl^- and K^+ influx, mitogen-activated protein kinases (MAPK) activation are elevated.

Upon activation of secondary messengers, there is the triggering of cellular defense mechanisms such as genetic expression of signaling molecules (e.g., jasmonate, salicylic acid, ethylene etc.,), expression of endogenous antioxidant enzymes (e.g., catalase, peroxidases etc.,), and *de novo* synthesis of transcription factors and mRNAs required for expression of the enzymes responsible for triggering the biosynthesis of secondary metabolites (Zhao et al., 2005; Baenas et al., 2014a).

Therefore, enhancement of phenolic biosynthesis can be achieved with elicitation under appropriate conditions, by recognizing and manipulating different control points of the plant physiology, towards the triggering and expression of transcription factors, mRNAs and key enzymes associated with the phenolic biosynthesizing pathway.

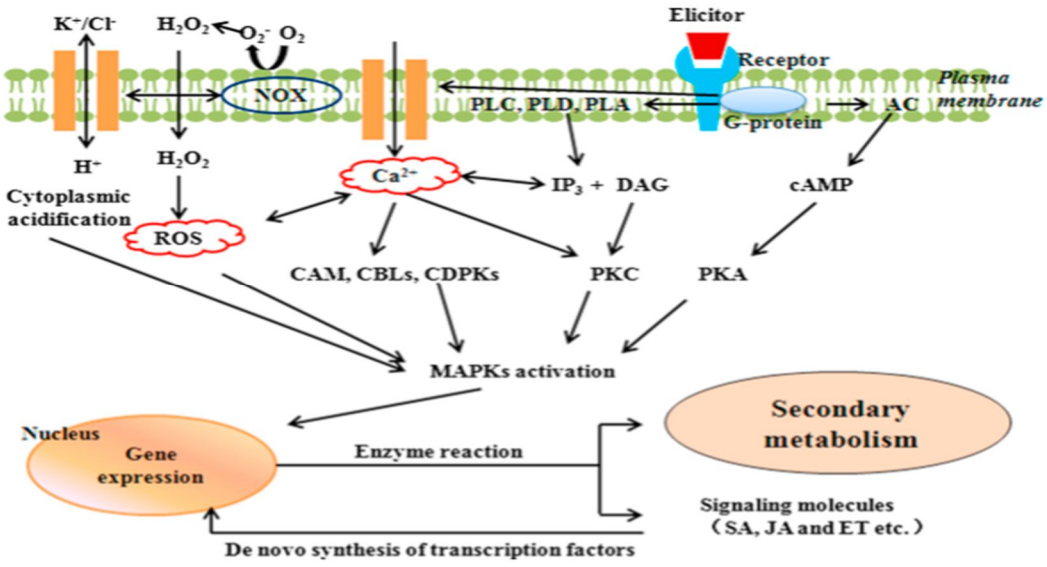


Figure 2.4: Mechanism of elicitation on synthesis of secondary metabolites. Abbreviations: NOX: nicotinamide adenine dinucleotide phosphate oxidase; O^{2-}: superoxide anion; H_2O_2: hydrogen peroxide; ROS: reactive oxygen species; PLA: phospholipase A; PLC: phospholipase C; PLD: phospholipase D; AC: adenylyl cyclase; cAMP: Cyclic Adenosine monophosphate; IP3: 1,4,5-triphosphate; DAG: diacylglycerol; CAM: calmodulin; CBLs: calmodulin-like proteins, CDPKs: calcium-dependent kinases; PKC: protein kinase C; PKA: protein kinase A; MAPKs: mitogen-activated protein kinase; ET: ethylene, SA: salicylic acid; JA: jasmonate. **Reference: Liu et al., 2019.**

According to authors such as Jeong et al. (2018); Thwe et al. (2014) and Liu et al. (2013), elicitors are able to influence phenolic accumulation through their stimulation of biosynthetic pathways (i.e., pentose phosphate, shikimate and phenylpropanoid pathways) and genetic expression or activities of key enzymes (e.g., phenylalanine ammonia-lyase, tyrosine ammonia-lyase, cinnamate hydroxylase etc.,) involved with these pathways. However, effects of elicitation on phenolic accumulation will depend on the plant characteristics (e.g., specie, cultivar, and developmental stage), environmental conditions and elicitor type (e.g., nature, dose, method of application, and treatment interval). Thus, the following sections provide examples of

documented biotic and abiotic elicitors, and their influence on accumulation of phenolic compounds and antioxidant capacity of diverse sprouts.

2.7.2 Classification of elicitors

2.7.2.1 Abiotic elicitors

2.7.2.1.1 Salts

Salts including NaCl, $NaNO_3$, $MnSO_4$, KNO_3, and Na_2SeO_3 are the most commonly reported abiotic elicitors with respect to phenolic accumulation (Owolabi et al., 2018). A previous work on NaCl (60 mM) elicitation of Thai rice resulted in significant increments of total phenolics, cyanidin-3-glucosides and antioxidant capacity, compared to their untreated forms (Daiponmak et al., 2010). Umnajkitikorn et al. (2013) also observed increased activity of antioxidant enzymes (catalase and ascorbate peroxidase) in the scavenging of ROS developed from NaCl elicited rice, compared to control treatments. Similarly, Swieca (2015) and Yuan et al. (2010) observed 36.64 and 16.67 % significant phenolic increases with 300 and 100 mM NaCl elicited lentil and radish sprouts, respectively, over the control.

Additionally, chickpea sprouts elicited with different concentrations of Na_2SeO_3 (1 and 2 mg/L) attained the optimum yields of total isoflavonoids, PAL activity and antioxidant capacity, compared to their unelicited forms (Guardado-Félix et al., 2017). According to these authors, 2 mg/L Na_2SeO_3 treated chickpea sprouts significantly enhanced total isoflavonoids, PAL activity and antioxidant capacity by 83, 56 and 33 %, respectively, compared to the control. Wang et al. (2016) tested $NaSeO_3$ (0, 3 and 6 mg/L) tolerance in peanut sprouts through the accumulation of phenolics as defense compounds. From the above experiment, peanut sprouts elicited with 6 mg/L of $NaSeO_3$ elicited the highest expression of phenylpropanoid triggering enzymes, that is PAL and C4H by 2.5 and 3.5 times, respectively, compared to the control. Correlating with these

enhanced enzymatic expressions, was the accumulation of caffeic, coumaric and ferulic acids with 6 mg/L $NaSeO_3$, which were significantly higher than the control by 62.50, 72.97 and 75 %, respectively.

2.7.2.1.2 Ultrasound

Ultrasonic powers applied in food treatments include sound waves of frequencies ranging from 20-100 kHz and sound intensity ranges of 10-1000 W/cm^2 (Piyasena et al., 2003). As reviewed by Rafina et al. (2019), during ultrasonication applied sound waves in a medium (usually water) are able to change the molecular structure of a material via a series of rarefaction and compression cycles. Following this, gas bubbles and cavitation are developed in areas of the material that were exposed to ultrasonic compressions, leading to changes in microstructure, microstreaming, cytosolic streaming and mass transfer (Soria and Villamiel, 2010).

In recent studies, there has been reports linking enhanced phenolic biosynthesis with ultrasonic treatments. A previous ultrasonic study (28, 45 and 100 kHz) by Yu et al. (2016) with three cultivars of peanut sprouts showed enhanced resveratrol contents. According to this study, compared to resveratrol levels of control treatments (2.55-23.43 µg/g), 100 kHz ultrasound treated sprouts significantly reported the highest resveratrol yield within ranges of 8.15-25.55 µg/g. Another study which investigated impact of ultrasound (100, 200 and 300 W) on accumulation of isoflavonoids with soybean sprouts also observed 39.13 and 96.91 % significant increments of daidzein and genistein, respectively, with 300 W ultrasound treatments compared to their untreated counterparts (Yang et al., 2015). From this same study, GABA levels were increased by 43.4% with 300 W ultrasound treatment, compared to control. Yu et al. (2016) also observed improved antioxidant capacity with ultrasonic treated Romaine lettuce.

2.7.2.1.3 Light and temperature

Manipulation of environmental factors such as light and temperature towards biosynthesis of phenolics in plant systems have also been evaluated by some research studies. The impact of light intensity and quality on phenolic biosynthesis has widely been studied with the use of light-emitting diodes (LEDs), based on the key role of light as carbon source during photosynthesis, as well as their use as signaling factors for plant growth and development (Aube et al., 2013; Lin et al., 2013).

Phommalth et al. (2008) evaluated the impact of dark, greenhouse, fluorescent, incandescent and ultraviolet lamps on isoflavone contents of soybean sprouts. From their study, elicitation of soybean sprouts with combined treatments of greenhouse (12 hr per day) and ultraviolet (40 min per day) resulted in 1.90-fold significant increases of isoflavone, compared to control. In another research with different light treatments (dark, red, blue, white, and red + blue) of Tartary buckwheat sprouts, optimum flavonoid content was elicited with red + blue treatments by 2%, compared to the control (Seo et al., 2015). A similar study by Qian et al. (2016) also investigated the influence of LEDs (red, blue, white and dark lights) on total phenolics, anthocyanin and antioxidant levels of Chinese kale sprouts. According to these authors, elicitation with blue LED resulted in 1.61, 1.75 and 18.16 % increases of antioxidant capacity, total phenolics and anthocyanins, respectively.

Additional to light elicitation, temperature manipulation has also shown phenolic enhancements. In an experiment with lentil sprouts, 2-day old sprouts were exposed to 4 and 40 ºC for 1 hr and sprouted afterwards at control temperature (25 ºC) for 3, 4 and 6 days (Swieca and Baraniak, 2014). From this work, 6-day lentil sprouts elicited with 40 ºC reported the highest levels of total phenolic acids and flavonoids, by being 18.14 and 32.80 % significantly higher

compared to untreated sprouts, linking their observations to the possible induction of the phenylpropanoid pathway against the negative effects of temperature shock.

2.7.2.2 Biotic elicitors

2.7.2.2.1 Proteins-based compounds

Protein compounds such as marine hydrolysates and lactoferrin have been reported to enhance accumulation of phenolic compounds when used as precursor substrates. Randhir et al. (2009) investigated influence of fresh protein hydrolysate and lactoferrin on phenolic (L-DOPA) contents of fava bean sprouts. From this study, fish protein hydrolysates and lactoferrin elicitors were found to increase stimulation of the pentose phosphate and phenylpropanoid pathways, which resulted in 20 and 40 % L-DOPA increases, respectively, compared to the control. L-DOPA (levo dihydroxy phenylalanine) is a phenolic compound that has been shown to reduce risks and effectively protect against Parkinson's disease.

2.7.2.2.2 Phytohormones and oligosaccharides

Plant hormones (e.g., methyl jasmonate, jasmonic acid, salicylic acid, melatonin and ethephon) and oligosaccharides (e.g., chitosan, sucrose, mannitol, glucose, fructose and chitooligosaccharide) have been used to positively influence accumulative levels of secondary metabolites (Baenas et al., 2016; Lee et al., 2013; Aguilera et al., 2015). Phytohormones act as important signals for genetic expression of enzymes and proteins when plants are exposed to wounding, fungal, bacterial and viral pathogens (Wang and Zheng, 2005). For instance, Pérez-Balibrea et al. (2011) investigated influence of different concentrations of salicylic acid (50, 100, 200 and 300 μM), methyl jasmonate (10, 25, 50 and 100 μM), and chitosan (0.01, 0.05, 0.1, 0.25

and 0.5 %) on phenolic levels of broccoli sprouts at days 3, 5 and 7. From this study, 100 µM salicylic acid reported the greatest level of total phenolics and antioxidant activity at day 7, which was 40% significantly higher compared to the control.

Other studies by Guo et al. (2011a) and Liu et al. (2013) showed enhanced phenolic contents with mannitol; sucrose and ethylene elicitations with broccoli and mung bean sprouts, respectively. Another study by Mendoza-Sanchez et al. (2016) investigated salicylic acid (0.1, 1 and 2 mM) and chitosan (0.7, 3.3 and 7 µM) effects on phenolic acids and antioxidant capacity of common bean (cv Dalia bean) sprouts. According to these authors, 1; 2 mM salicylic acid elicited the highest levels of coumaric (8.5-fold), salicylic (115-fold), gallic (25-fold), caffeic (1.7-fold) acids and antioxidant capacity (37%), compared to control sprouts.

2.7.2.2.3 Amino acids

Research has documented data on positive results of amino acids such as L-phenylalanine, L-tyrosine, tryptophan, and DL-methionine on phenolic accumulations (Limon et al., 2014; Dueñas et al., 2015; Swieca, 2016; Peñas et al., 2015). The principle of their action is based on their use as substrates for some precursors associated with the phenolic biosynthesizing pathways. For example, L-phenylalanine and L-tyrosine are respective substrates for phenylalanine ammonia-lyase and tyrosine ammonia-lyase, where they are reduced by these enzymes into intermediate metabolites needed for subsequent generation of various phenolic compounds associated with the phenylpropanoid pathway (Mendoza-Sanchez et al., 2016). Also, glutamic acid serves as a substrate for proline biosynthesis, which has been linked to phenolic biosynthesis through the triggering of the pentose phosphate pathway (i.e., the pathway

responsible for providing substrates linked to the triggering of the shikimate and phenylpropanoid pathways) (Burguieres et al., 2007).

A study by Limon et al. (2014) which investigated glutamic acid (5 mmol/L) elicitation on kidney bean sprouts revealed that, 8-day glutamic acid treatments elicited the highest level of soluble phenolics (6.06 mg GAE/g), compared to the control (5.22 mg GAE/g). However, this study observed 13.35% reduced antioxidant capacity with glutamic acid treatment compared to the control, attributing their observation to the possible activities of guaiacol peroxidase (the enzyme responsible for mobilizing free phenolics towards lignification for structural development), and polyphenol oxidases (the enzyme responsible for mobilizing monophenols into quinones and subsequent browning).

Another work by Swieca et al. (2014) which investigated the influence of phenylalanine and tyrosine on phenolic and antioxidant activity of lentil sprouts detailed the highest DPPH, ORAC and metal ion chelating capacities with phenylalanine elicitation, at significant levels of 29, 28.43 and 23 %, respectively, compared to the control. These authors attributed their observations to significant phenolic increments and PAL activities with phenylalanine elicitation, than the control treatments. Comparably, Liu et al. (2013) observed 112.50, 136.05 and 100.00 % significant increments of eriodictyol-*O*-acylhexoside, quercetin 3-*O*-glucoside and kaempferol 3-*O*-acetylhexoside, respectively, with 5 mM glutamic acid elicited kidney bean sprouts, compared to their control counterparts.

2.7.2.2.4 Pathogens

This group is regarded as the earliest biotic elicitor. Upon association with plants, pathogens are able to release enzymes and cell wall fragments that are recognized by plant cells

to induce the production of defence compounds, popularly known as phytoalexins, upon association with whole plants or plant cell cultures. Manero et al. (2003) observed increased levels of cardenolides in *Digitalis lanata* elicited with strains of rhizobacteria. Ramos-Solano et al. (2010), also investigated nine strains of rhizobacteria with soybean. From this study, levels of daidzein and genistein were significantly increased upon elicitation with rhizobacteria. In another study by Zhao et al. (2001), concentrations of beta-thujaplicin were increased in *Cupressus lusitanica* by fungal elicitation. Also, Broeckling et al. (2005) reported beta-amyrin increases in *Medicago truncatula* upon yeast elicitation.

CONNECTING STATEMENT TO CHAPTER III

Chapter II of this dissertation provides a review of the literature on biochemical composition of common bean. From this review it was noted that, the health benefits of common bean are due to the presence of macronutrients, micronutrients and secondary metabolites such as phenolic compounds. However, the nutritional value of common bean is limited due to the presence of antinutritional compounds. Thus, the need for common bean processing before consumption. Among processing methods, it was noted that sprouting was more efficient in protecting heat-sensitive nutrients, presenting stored nutrients in bioavailable forms, and enhancing accumulation of phenolics.

Investigation on the impact of sprouting on nutrient-density and nutraceutical properties of five common bean cultivars was detailed in Chapter III. Three nutrient-density indexes including Nutrient Rich Food 9.3 (NRF9.3), Ratio of Recommended to Restricted nutrients (RRR) and SAIN LIM were used to assess impact of sprouting on nutrient-density profiles of common beans. Phenolic contents and antioxidant capacities of sprouted beans were also evaluated and compared to their untreated forms. This chapter is under review for publication in the Journal of Food Science and Technology as follows:

Ampofo, J. O., Kwofie, E., M. and Ngadi, M. (2019). Impact of sprouting on nutrient-density and nutraceutical properties of common bean (*Phaseolus vulgaris)*. Journal of Food Science and Technology. (Manuscript ID, JFST-D-19-01906).

CHAPTER III

Impact of Sprouting on Nutrient Density and Nutraceutical Properties of Common Beans (*Phaseolus vulgaris*)

3.1 Abstract:

The aim of this study was to investigate how sprouting affects nutrient-density and nutraceutical properties of common beans. Common beans were analyzed for nutrients, dietary fiber, anti-nutrients, polyphenols and antioxidative properties. Nutrient density scores were estimated by the Nutrient Rich Food Index (NRF9.3), Ratio of Recommended to Restricted (RRR) Index and SAIN LIM index. Compared to raw beans, sprouted bean cultivars were richer in nutrients, dietary fiber, polyphenols and antioxidant potential. NRF9.3, RRR, and SAIN LIM scores for sprouted beans ranged between 235.12-303.93, 11.72-15.17, and 11.08-14.51, respectively, whereas raw beans reported ranges of 188.19-215.22, 9.38-10.8, and 8.84-10.25, respectively. Indexes of total polyphenols, total antioxidant and anti-nutrients after sprouting also ranged within 1.72-3.09, 1.10-1.76 and 0.69-0.40, respectively, whereas their unsprouted forms reported 1.00 for all respective indexes. Thus, sprouted common beans can serve as ingredients for the formulation of healthy food products.

3.2 Introduction

According to the USDA, nutrient-dense foods are those that supply significant amounts of nutrients with minimum calories (Gregory et al., 2009). Dietary guidelines such as the Canadian food guide, dietary guidelines for Americans and MyPlate recognize nutrient-dense foods as among specific food groups such as fruits, vegetables, whole pulses, whole grains, nuts and seeds.

Common beans (*Phaseolus vulgaris*), an example of a pulse, are vastly grown worldwide and form a major component of diets in different cultures. Common beans are rich sources of plant protein, minerals, dietary fiber, and amylose (Roy et al., 2010). In addition to its nutritional value, common beans are also rich in nutraceutical compounds such as polyphenols (e.g., flavonoids, phenolic acids etc.,), which have been reported to offer health benefits such as protection against risks of chronic diseases (e.g., type 2 diabetes, and obesity, etc.) (Hayat et al., 2014). Thus, common beans when added to daily diets can serve as a nutrient-rich food intervention among developed and developing populations. Notwithstanding their health benefits, common beans contain antinutritional compounds (e.g., tannin and enzyme inhibitors), which decrease bioavailability of nutrients (Mendoza-Sánchez et al., 2016). Thus, to increase their nutrient-density, reduction or removal of these negative compounds is imperative before consumption.

In recent years, the modern-day consumer demands low-processed foods that offer a duo advantage, that is, being nutrient-dense and having nutraceutical value at the same time. Several studies have reported the capacity of sprouting in reducing antinutritional compounds and improving the nutritional value of pulses (Pajak et al., 2014). Changes effected by sprouting have been linked to its activation of endogenous enzymes that break down storage molecules into their

simpler bio-accessible and bio-available forms. Furthermore, genes and transcription factors responsible for triggering of polyphenolic synthesis (e.g., phenylpropanoid pathway) are activated during sprouting, leading to their *novo* synthesis in sprouts, compared to dry seeds (Sibian et al., 2017). However, to our knowledge, there's no report on how sprouting influences nutrient density scores of common beans. Consequently, the aim of this study was to evaluate the effect of sprouting on nutrient-density and nutraceutical properties of five common bean (*Phaseolus vulgaris*) cultivars.

3.3 Materials and Methods

3.3.1 Samples

Five common bean (*Phaseolus vulgaris*) cultivars including pinto, kabulengeti, mandondo, misiska and sugar beans were used in this study. Pinto bean was purchased from Bulk Barn, Montreal, Canada. Kabulengeti and mandondo beans were provided by the Council for Scientific and Industrial Research Center of Zambia, whereas misiska and sugar beans were obtained from the Council for Scientific and Industrial Research Center of Malawi.

3.3.2 Treatments

3.3.2.1 Raw beans

Bean seeds were hand cleaned to remove impurities and damaged seeds, grounded into a flour by a Cyclone Sample Mill (UDY Corp., Fort Collins, CO, USA) fitted with a 1 mm screen, and stored at 4 ºC until analysis.

3.3.2.2 Sprouting

Sprouting was done as reported by Mubarak (2005). Bean seeds (100 g) were disinfected with 1% sodium hypochlorite (500 mL) for 20 min and rinsed with NAYA mineral water (NAYA Inc, Quebec, Canada) until neutral pH was attained. Next, the seeds were soaked in NAYA mineral water (1:3 w/v) at 25 ºC for 24 h, then drained and transferred into sprouting jars. Seeds were rinsed with NAYA mineral water (25 mL), three times daily at intervals of 6 h during the sprouting process. Sprouting was performed in darkness at 25 ºC for 7 days in an incubator. Afterwards, beans sprouts were oven dried at 50 ºC for 24 h, then milled using a Cyclone Sample Mill (UDY Corp., Fort Collins, CO, USA) fitted with a 1 mm screen, and stored at 4 ºC until analysis.

3.3.3 Nutrient analysis

3.3.3.1 Analysis of crude fat, crude protein and energy

AOAC (2005) procedures were used to determine crude fat (920.39), and crude protein (992.15). Energy content was determined by multiplying percentages of crude protein, crude fat and carbohydrates by their respective Atwater factors, which are 4, 9 and 4, respectively.

3.3.3.2 Determination of minerals

K, Na, Ca, Mg, and Fe were analyzed according to the method of Parkinson (1975). Approximately, 160 mg of sample flour was digested in 4.4 mL of a solution of sulfuric acid and hydrogen peroxide (1.2:1, v/v) at 340 °C for 3 h. After digestion, the obtained mixture was diluted to 100 mL with distilled water, and minerals were assessed by atomic absorption spectrometry.

3.3.4 Antinutritional compounds

3.3.4.1 Tannins

Tannin was determined spectrophotometrically as described by Broadhurst and Jones (1978). The amount of tannins was calculated as a catechin equivalent in mg/g of sample flour.

3.3.4.2 Oxalate

Oxalate was determined as described by Shadan et al. (2014). Seventy-five mL of 3M H_2SO_4 were added to 1 g of sample flour and extracted for 1 h and then filtered. Exactly 25 mL of the filtrate was heated to 80-90°C and titrated against 0.02 M $KMnO_4$ solution to an end-point of a faint pink color. Analyses were performed in triplicate. Oxalate concentration of sample was calculated from the equation below and expressed in mg/g of sample flour:

1mL 0.02 M permanganate = 0.006303 g oxalate Eq. (3.1)

3.3.4.3 Trypsin inhibitor activity

Trypsin inhibitor activity was determined as reported by Swieca (2015) with Nα-Benzoyl-DL-Arginine-p-Nitroanilide (BAPNA) as substrate. Trypsin inhibitor activity was expressed in trypsin units inhibited by 1 mg of sample (TUI/mg sample).

3.3.5 Nutraceutical analysis

3.3.5.1 Determination of dietary fiber

Dietary fiber was determined according to the method of AOAC (2002). One gram of sample flour was added to 50 mL phosphate buffer (pH 6), placed in a water bath at 100°C, and 0.1 mL of α-amylase solution was added and incubated (30 min) under continuous stirring.

Obtained slurry was cooled, and 0.1 mL of protease was added and incubated (60°C, 30 min). Afterwards, pH of obtained slurry was adjusted to 4, and samples were incubated (60°C, 30 min), and 0.3 mL of amyloglucosidase was added. Incubation was continued for 30 min under constant stirring. Afterwards, insoluble fractions were isolated by filtration, and soluble fractions were precipitated with 95% of ethanol (1:4 ratios). Both residues were washed three times with 10 mL distilled water and dried (90°C, 2 h). Dried residues and dried ethanol precipitates correspond to insoluble dietary fiber (IDF) and soluble dietary fiber (SDF), respectively. Determinations of residual ash and protein were carried out in both residues for possible corrections. Total dietary fiber (TDF) was calculated as the sum of IDF and SDF.

3.3.5.2 Phenolic compounds and antioxidant activities

3.3.5.2.1 Extraction of phenolic compounds

Phenolic compounds were extracted as outlined by Marathe et al. (2011). One gram of sample flour was extracted with 15 mL of 80% aqueous methanol (V/V). Afterwards, the mixture was centrifuged (5000 rpm, 10 min), supernatants filtered, methanol evaporated and obtained extracts were freeze-dried, and used for analysis.

3.3.5.2.2 Determination of phenolic compounds

Total phenolic acids, anthocyanins and tartaric esters were determined as described by Mazza et al. (1999). Amounts of total phenolic acids, anthocyanins and tartaric esters were calculated as catechin, cyanidin-3-glucoside and caffeic acid equivalents in mg/100g of sample flour. Total flavonoid content was measured as described by Hairi et al. (1991). Total flavonoid was calculated as rutin equivalent in mg/100g of sample flour.

3.3.5.3 Antioxidant activities

3.3.5.3.1 Estimation of DPPH free radical scavenging activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging capacity was carried out by the method of Akond et al. (2011) at different phenolic extract concentrations (250, 350, 450 and 550 µg/mL). Percentage antioxidant activity was calculated using the equation below:

$$\% \text{ Scavenging activity} = \frac{A_{control} - A_{test}}{A_{control}} \times 100 \qquad \text{Eq. (3.2)}$$

3.3.5.3.2 Estimation of ABTS scavenging activity

ABTS scavenging activity was determined according to the method of Re et al. (1999) at phenolic extract concentrations of 250, 350, 450 and 550 µg/mL. ABTS scavenging capacity was calculated using Eq. 3.2.

3.3.5.3.3 Estimation of Fe^{2+} chelating activity

Chelating of ferrous ions by extracts was estimated by the method of Dinis et al. (1994) with phenolic extract concentrations of 250, 350, 450 and 550 µg/mL. Ferrous ion chelating capacity was calculated using Eq. 3.2.

3.3.6 Theoretical contribution of sprouting to common beans nutrient-density and nutraceutical quality

3.3.6.1 Calculation of Nutrient density indexes (NDI)

To calculate Nutrient Density indexes, reported nutrient values of raw and sprouted common beans from this study were integrated into the Nutrient Rich Food Index (NRF9.3)

(Sluik et al., 2015), Ratio of Recommended to Restricted Nutrient Density Index (RRR) (Drewnowski, 2005) and the SAIN LIM index (Tharrey et al., 2017).

The NRF9.3 was calculated as the sum of the percentage daily values (% DV) of nine nutrients to encourage (NR9) minus the sum of the percentage daily values (%DV) of three nutrients to limit (LIM3), based on 100 g of beans flour, as shown below:

$$NRF9.3 = \Sigma(\%\text{DV protein} + \%\text{DV fiber} + \%\text{DV vitamin A} + \%\text{DV vitamin C} + \%\text{DV vitamin E} + \%\text{DV calcium} + \%\text{DV iron} + \%\text{DV potassium} + \%\text{DV magnesium}) - \Sigma(\%DV\text{ saturated fatty acids} + \%\text{DV sodium} + \%\text{DV added sugar}) \quad \text{Eq. (3.3)}$$

RRR Nutrient Density Index was calculated as the mean percentage daily values (% DV) of six recommended nutrients divided by the mean percentage daily values (%DV) of five restricted nutrients, based on 100 g of beans flour, as depicted below (Eq. 3.4):

$$\frac{(\Sigma\%\text{DV protein} + \%\text{DV fiber} + \%\text{DV vitamin A} + \%\text{DV vitamin C} + \%\text{DV calcium} + \%\text{DV iron})/6}{(\Sigma\%\text{DV calories} + \%\text{DV sugar} + \%\text{DV saturated fat} + \%\text{DV cholesterol} + \%\text{DV sodium})/5} \quad \text{Eq. (3.4)}$$

SAIN

$$\frac{\frac{\text{Proteins}\ (\frac{\text{g}}{\text{energy kcal}})}{65} + \frac{\text{Fibers}\ (\frac{\text{g}}{\text{energy kcal}})}{25} + \frac{\text{Vit C}\ (\frac{\text{mg}}{\text{energy kcal}})}{110} + \frac{\text{Fe}\ (\frac{\text{mg}}{\text{energy kcal}})}{12.5} + \frac{\text{Ca}\ (\frac{\text{mg}}{\text{energy kcal}})}{900}}{5} \times 100 \quad \text{Eq. (3.5)}$$

LIM

$$\frac{\frac{\text{Sodium}\ (\frac{\text{mg}}{100\text{g}})}{3157^{*}} + \frac{\text{Saturated fatty acids}\ (\frac{\text{g}}{100\text{g}})}{22^{**}} + \frac{\text{Added sugar}\ (\frac{\text{g}}{100\text{g}})}{50^{**}} \times 100}{3} \quad \text{Eq. (3.6)}$$

3.3.6.2 Total antinutritional index (ANI)

One factor limiting nutrient-density of common beans is the presence of antinutritional factors. To our knowledge, no nutrient-density model has included the factor of antinutritional compounds. Three antinutritional compounds were integrated into the proposed model of Swieca et al. (2014) to obtain the overall antinutritional index (ANI). ANI was calculated as the sum of the relative antinutrients (RAN) divided by the number of antinutrients (n).

$$\text{Relative antinutrients index (RAN)} = \frac{\Sigma RI_{an}}{n} \quad \text{Eq. (3.7)}$$

$$\text{where } RI_{an} = \frac{A_s}{A_r} \quad \text{Eq. (3.8)}$$

Where A_s = concentration of an antinutritional compound determined in common bean sprouts, A_r = concentration of an antinutritional compound determined in raw common beans.

3.3.6.3 Total phenolic compound index (PI)

Four phenolic compounds were integrated into the model of Swieca et al. (2014) to obtain the overall total phenolic index (PI). PI was calculated as the sum of the relative phenolic compounds (RP) divided by the total number of investigated phenolic compounds (n).

$$\text{Relative phenolic index (RP)} = \frac{\Sigma \mathrm{RP_i}}{\mathrm{n}} \quad \text{Eq. (3.9)}$$

RP_i was determined as follows:

$$RP_i = \frac{P_s}{P_r} \quad \text{Eq. (3.10)}$$

Where P_s = concentration of a phenolic compound determined in common bean sprouts, P_r = concentration of a phenolic compound determined in raw common beans.

3.3.6.4 Total antioxidant activity index (AI)

Due to the complexity of food systems, no single method can describe their antioxidant activity. Thus, to further evaluate antioxidant capacity, the total antioxidant activity index (AI) proposed by Swieca et al. (2014) was adopted, by using three antioxidant methods. Total antioxidant activity index (AI) was calculated as the sum of the relative antioxidant activities (RA) for each antioxidant chemical method divided by the number of methods (n).

$$AI = \frac{\Sigma RA_n}{n} \qquad \text{Eq. (3.11)}$$

RA was calculated as follows:

$$RA = \frac{A_S}{A_C} \qquad \text{Eq. (3.12)}$$

Where: A_s denotes the antioxidant activity of common bean sprout for a tested method, A_c denotes the antioxidant activity of raw common beans (control) determined for the tested method.

3.4 Results and discussion

3.4.1 Changes in nutrient contents

Quantitative levels of macro and micronutrients are important factors in determining the nutrient-density of a food. Common beans variety and sprouting significantly ($P < 0.05$) affected levels of crude fat, crude protein, minerals and energy. Crude fat levels in raw cultivars ranged within 1.05-1.95 g/100 g, whereas the values were decreased in sprouted cultivars in the range from 0.82-1.79 g/100 g (Table 3.1). Congruent with this study, Singh et al. (2017) also observed decreasing crude fat contents in 2-day sorghum sprouts (1.61%), compared to their raw form (3.03%). Reductions in crude fat could be due to activity of lipases, in providing energy for the

sprouting process. Presence of excess fat in diets has been correlated to chronic diseases. Therefore, addition of common bean sprouts with reduced crude fat contents to daily diets will help minimize overall energy density of diet.

Also, crude protein levels were significantly ($P < 0.05$) increased after sprouting by 22.73, 17.41, 14.86, 13.02 and 5.97 % for pinto, mandondo, kabulengeti, sugar and misiska beans, respectively (Table 3.1). During sprouting, imbibition of water by common bean seeds induces gibberellic acid activities, triggering endogenous proteases to breakdown storage proteins into nitrogen needed for embryonic growth and early structural development. Proteins in diets have diverse advantages, ranging from body development to growth. Sprouted common beans from this study with higher protein levels can be complemented with cereal and grain foods, as a natural dietary intervention against protein-energy imbalances associated with cereals and grains diets.

From Table 3.1 Ca and Mg levels were significantly higher for sprouted common beans, compared to their raw counterparts, ranging between 1.45-2.22 and 9.92-14.18 mg/g, respectively. Increase in Ca and Mg contents of common bean sprouts may be attributed to the presence of calcium and magnesium salts in rinsing water used during the sprouting process. Calcium (Ca) and Mg improved common bean sprouts can be used to formulate diets for rural populations in Sub Saharan Africa where access to dairy foods as the main dietary source of Ca, and Mg are limited. Supporting our observation is the work of Mubarak (2005) similarly reporting increased K, Ca, and Mg levels in mung bean sprouts.

Table 3.1: Nutritional, energy and antinutritional composition of raw and sprouted common bean cultivars (dry weight basis)

Analyte	Pinto		Sugar		Kabulengeti		Mandondo		Misiska	
	Raw	Sprout	Raw	Sprout	Raw	Sprout	Raw	Sprout	Raw	Sprout
Nutrient										
Energy (kcal)	354.87±2.95^{de}	360.07±0.61^{bcd}	358.06±4.42^{cde}	368.77±0.26^{a}	364.29±2.35^{abc}	365.24±0.68^{ab}	353.75±0.44^{de}	358.36±2.89^{cde}	352.74±0.77^{e}	365.90±2.49^{ab}
Protein (g/100g)	26.47±0.44^{cd}	32.48±0.44^{a}	22.09±0.44^{d}	28.39±0.44^{bc}	24.38±0.44^{d}	28.23±0.44^{bc}	24.66±0.44^{d}	28.95±0.44^{b}	27.49±0.44^{bc}	29.17±0.44^{b}
Crude fat (g/100g)	1.85±0.00^{ab}	1.32±0.01^{ab}	1.95±0.01^{a}	1.79±0.00^{ab}	1.38±0.01^{ab}	0.95±0.00^{ab}	1.05±0.00^{ab}	0.83±0.00^{b}	1.24±0.00^{ab}	0.82±0.00^{b}
Ca[1] (mg/g)	1.08±0.19^{de}	2.22±0.04^{a}	0.95±0.11^{e}	1.78±0.05^{bc}	1.07±0.04^{de}	2.19±0.22^{ab}	0.74±0.07^{e}	1.45±0.05^{cd}	0.92±0.04^{e}	1.49±0.01^{cd}
Fe (mg/g)	0.08±0.00^{a}	0.11±0.06^{a}	0.15±0.05^{a}	0.16±0.05^{a}	0.16±0.01^{a}	0.06±0.02^{a}	0.14±0.00^{a}	0.09±0.03^{a}	0.07±0.01^{a}	0.12±0.00^{a}
Mg (mg/g)	1.46±0.08^{cd}	1.96±0.04^{a}	1.34±0.05^{d}	1.90±0.02^{a}	1.51±0.11^{cd}	1.99±0.10^{a}	1.30±0.01^{d}	1.64±0.02^{bc}	1.44±0.03^{cd}	1.86±0.03^{ab}
K (mg/g)	11.45±0.33^{bc}	11.84±0.18^{bc}	11.50±0.25^{bc}	14.18±1.20^{a}	11.50±0.74^{bc}	9.92±0.54^{c}	10.55±0.35^{c}	11.43±0.19^{bc}	11.90±0.13^{bc}	13.03±0.00^{ab}
Na (mg/g)	0.00±0.00^{c}	0.02±0.02^{bc}	0.00±0.00^{c}	0.04±0.00^{bc}	0.06±0.03^{ab}	0.09±0.00^{a}	0.02±0.01^{bc}	0.03±0.01^{bc}	0.01±0.01^{c}	0.04±0.00^{bc}
Anti-nutrients										
TIA (TIU/mg)	5.58±0.00^{a}	3.11±0.00^{h}	4.87±0.00^{d}	3.75±0.00^{f}	5.29±0.02^{b}	2.70±0.00^{j}	5.09±0.00^{c}	3.57±0.00^{g}	4.58±0.02^{e}	2.82±0.00^{i}
Tannin (mg/g)	0.14±0.00^{d}	0.07±0.00^{f}	0.16±0.02^{cd}	0.09±0.00^{ef}	0.19±0.01^{b}	0.11±0.00^{e}	0.29±0.01^{a}	0.08±0.00^{f}	0.16±0.01^{c}	0.09±0.00^{ef}
Oxalate (mg/g)	36.53±1.09^{d}	22.70±0.00^{f}	38.63±1.44^{d}	28.80±0.69^{e}	52.93±2.27^{a}	44.10±0.00^{c}	45.37±1.25^{bc}	35.10±2.52^{d}	48.73±1.94^{ab}	0.43±0.75^{g}

Mean values in a row that do not share the same superscripts are significantly different from each other when $P < 0.05$. Ca - Calcium; Fe - Iron; Mg - Magnesium; K - Potassium; Na – Sodium; TIA: Trypsin inhibitor activity

Compared to raw beans (range 352.74-364.29 kcal), there were significant increases for mean values of energy observed for sprouts of sugar (368.77 kcal) and misiska (365.90 kcal) beans, compared to other investigated common beans. Most significantly, this observation was due to the elevated levels of crude protein among sprouted common beans, and not due to carbohydrate and fat contents. Low protein diets, accompanied with high energy levels is the main dietary cause for developing a chronic disease, thus, sprouted common beans from this study can be recommended as dietary interventions needed to curtail the rising protein-energy insecurities among modern day consumers. Overall, nutritional changes observed in common beans were significantly dependent on sprouting and variety, due to variations in variety genetic make-up, concentrations and activation rates of endogenous hormones and enzymes responsible for metabolic processes along the sprouting process for each bean type.

3.4.2 Antinutritional compounds and total antinutritional index

Effect of sprouting on antinutrients composition of common beans are presented in Table 3.1. Trypsin inhibitor activity (TIA) ranged between 4.58-5.48 TIU/mg and 2.70-3.75 TIU/mg, for raw and sprouted common bean cultivars, respectively. The highest % reduction was observed for sprouted kabulengeti (49.05%), followed by pinto (43.19%), misiska (38.43%), mandondo (29.90%) and sugar beans sprouts (22.98%). Due to the proteinaceous nature of TIA, they can be hydrolyzed by proteases during the sprouting process, thus accounting for our observation.

After 7 days of sprouting, tannins reduced by 41.67, 46.78, 47.01, and 74.41 % for sprouts of sugar, pinto; kabulengeti, misiska and mandondo, respectively. Because tannins are mostly distributed in the seed coat, the observed reductions in sprouted beans could be due to

their leaching into the soaking water (Gemede and Ratta 2014). Concentrations of oxalate was tremendously reduced after sprouting of common beans, with levels in raw and sprouted forms ranging between 36.53-52.93 and 0.43-44.10 mg/g, respectively. Oxalate was reduced by 16.69, 22.63, 25.45, 37.86, and 99.11 % for sprouts of kabulengeti, mandondo, sugar, pinto and misiska beans, respectively. Oxalate changes in beans sprouts could be due to their oxidation by oxalate oxidase, the enzyme responsible for oxidation of oxalates into carbon dioxide and hydrogen peroxide.

Overall, sprouting significantly reduced total antinutritional index (ANI) of common beans, with the lowest ANI reported for sprouted misiska (0.40), followed by pinto (0.56), mandondo (0.58), kabulengeti (0.64) and sugar (0.69) beans, in comparison to their raw forms (1.00) (Fig. 3.1). Decreased ANI of sprouted common beans will help enhance nutrient-density of common beans by improving digestion and bio-availabilities of protein, amino acids, minerals and vitamins. Thus, reduced ANI coupled with elevated protein contents in common beans sprouts from this study can be used for formulation of nutrient-dense foods targeted for consumers who cannot consume whole foods due to their bulkiness, for instance among infants, children and the aging population.

Additionally, significant reductions of trypsin inhibitor levels observed in common bean sprouts may help protect consumers against risks of developing pancreatic hypertrophy, because less digestive enzymes may be demanded from the pancreas during sprout digestion, due to low TIA. Furthermore, ANI reported for sprouted beans may signify their possible protection against risk of type-2 diabetes and proliferation of cancerous cells, through their amylase inhibition (i.e., by tannins) and protease inhibition (i.e., TIA) mechanisms, respectively.

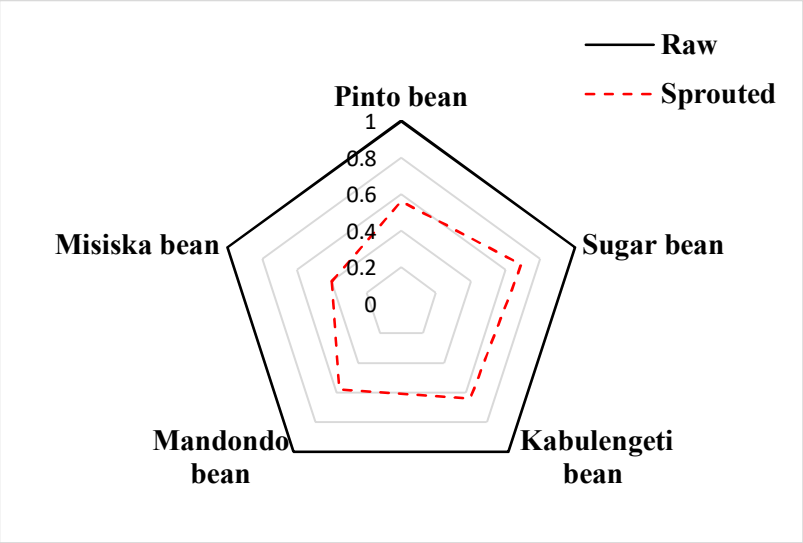


Figure 3.1: Radar chart showing mean values for total antinutritional index of 5 cultivars of raw and sprouted common bean. Data represent mean values ± SD of triplicates

3.4.3 Dietary fiber

As shown in Table 3.2, insoluble dietary fiber (IDF) constituted about 90% of TDF in all common bean sprouts. Compared to raw common beans, increasing order of IDF in sprouts was as follows: misiska (35.48 to 53.56 g/100 g), mandondo (37.22 to 52.11 g/100 g), pinto (30.80 to 47.33 g/100 g), sugar (34.39 to 46.25 g/100 g), and kabulengeti (30.62 to 38.22 g/100 g) beans. A similar trend of increases was observed for soluble dietary fiber (SDF), with the lowest (5.72 g/100 g) and highest (7.42 g/100 g) mean values observed for sprouts of sugar and misiska beans, respectively. Overall, total dietary fiber (TDF) after sprouting increased from 34.43 to 44.32 g/100 g for kabulengeti, whereas in sugar bean sprouts, TDF increased from 38.44 to 51.97 g/100 g. TDF increased in pinto sprouts from 34.49 to 53.61 g/100 g, while in mandondo and misiska sprouts, we observed increments from 41.41 to 58.77 and 39.76 to 60.97 g/100 g, respectively.

Table 3.2: Dietary fiber content of raw and sprouted common bean cultivars (g/100 g, dry weight basis)

Cultivar	IDF[1]	SDF	TDF	IDF:SDF
Raw				
Pinto	30.80±1.49^{d}	3.69±0.06^{g}	34.49±1.44^{e}	8.36
Sugar	34.39±0.46^{cd}	4.05±0.03^{efg}	38.44±0.44^{d}	8.49
Kabulengeti	30.62±0.31^{d}	3.81±0.04^{fg}	34.43±0.28^{e}	8.04
Mandondo	37.22±1.41^{c}	4.19±0.04^{ef}	41.41±1.45^{cd}	8.88
Misiska	35.48±0.36^{c}	4.28±0.04^{e}	39.76±0.41^{d}	8.29
Sprouted				
Pinto	47.33±3.58^{b}	6.28±0.06^{bc}	53.61±3.64^{b}	7.54
Sugar	46.25±0.72^{b}	5.72±0.14^{d}	51.97±0.59^{b}	8.09
Kabulengeti	38.22±0.57^{c}	6.10±0.07^{cd}	44.32±0.56^{c}	6.26
Mandondo	52.11±0.96^{a}	6.66±0.17^{b}	58.77±0.99^{a}	7.82
Misiska	53.56±1.29^{a}	7.42±0.39^{a}	60.97±0.93^{a}	7.21

Mean values with different superscripts in a column for a bean type (Raw, R or Sprouted, S) are significantly different from each other at $P < 0.05$. [1]IDF – Insoluble dietary fiber; SDF – Soluble dietary fiber; TDF – Total dietary fiber

During sprouting, enzymes such as cellulase, and pectinase are activated for breaking down cellulose and pectin cell wall components, leading to increments in cellulosic glucose, arabinose, xylose etc. Dietary fiber has been reported in literature to serve as nutraceutics, offering health benefits such as being anticarcinogenic, antidiabetic, antihyperlipidemic etc., (Hayat et al., 2014).

From this study, consumption of common beans sprouts with increased SDF fractions will help delay starch digestion, and induce early satiation, thus helping control risks of obesity, diabetes and weight gain. Also, improved SDF fractions will help increase concentrations of short-chain fatty acids (e.g., propionate), promoting survival of healthy intestinal microbes (e.g., *Lactobacillus acidophilus*). Enhanced IDF fractions observed in common bean sprouts may also provide an additional advantage of helping reduce risks of diverticulosis, through contributions to bulkiness to stool and free bowel movement.

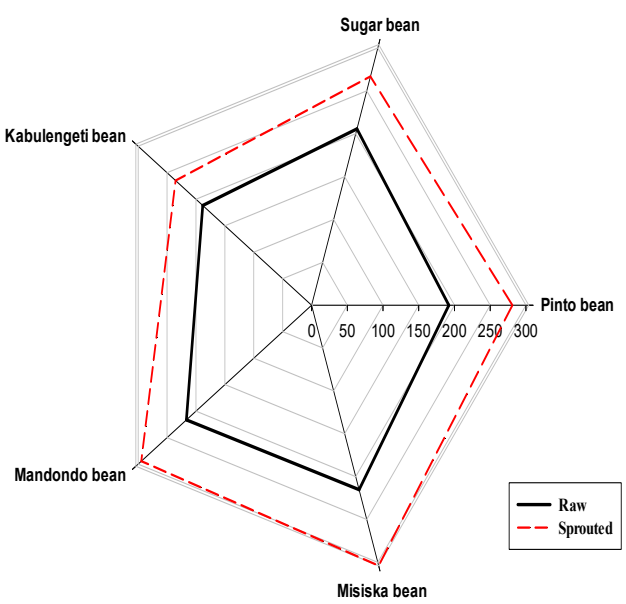
3.4.4 Nutrient density profiling

From this study, variety and sprouting significantly ($P < 0.05$) influenced nutrient density indexes of common beans, as shown in Figure 3.2 a-c. Comprehensively, sprouted common beans were significantly ($P < 0.05$) nutrient-dense compared to raw forms, with NRF9.3 and RRR scores ranging between 235.12-303.93 and 11.72-15.17, respectively, whereas their raw forms reported ranges of 188.19-215.22 and 9.38-10.8, respectively. After sprouting, we observed NRF9.3 indexes increasing by 24.94, 29.73, 36.02, 41.22 and 46.322 % for kabulengeti, sugar, mandondo, misiska and pinto beans, respectively. However, we observed significant ($P < 0.05$) variations for NRF9.3 scores among sprouted common bean varieties. For instance, sprouted misiska and kabulengeti reported the highest and lowest NRF9.3 scores, and were significantly different from each other. Similarly, for RRR index, significant ($P < 0.05$) higher scores were observed for sprouted misiska with the significant highest RRR score of 15.17, followed by mandondo (14.70), pinto (14.03), sugar (13.30) and kabulengeti (11.72) beans, respectively, compared to their raw forms which showed scores of 10.74, 10.80, 9.58, 10.26 and 9.38, respectively. However, variations existed among sprouted varieties. For example, sprouted mandondo was significantly higher, when statistically compared to sprouted sugar bean. SAIN scores for sprouted pinto (13.3), sugar (12.67), kabulengeti (11.08), mandondo (14.04) and misiska (14.51) beans were similarly significantly ($P < 0.05$) higher than raw forms. Furthermore, the trend of significant variations among sprouted common bean varieties was also observed for SAIN scores.

Combining NRF9.3, RRR and SAIN LIM nutrient density indexes of sprouts, misiska bean showed the highest mean score of 111.20, whereas kabulengeti bean showed the lowest mean score of 85.97. NRF9.3, RRR and SAIN scores reported for this study signify that, beans

in their sprouted forms are significantly nutrient-dense, compared to their raw forms, thus can contribute substantial amounts of key nutrients when added to daily diets. This observation can be attributed to the high protein and fiber contents reported for all common bean sprouts cultivars, coupled with low levels of sodium, sugar and saturated fat. According to the FDA, "healthy" foods are those that contain at least 10% DV per reference amount of one or more of six beneficial nutrients (Zanovec et al., 2011). Hence, based on the above FDA recommendation, NRF9.3 and RRR indexes, a serving of 100 g of sprouted kabulengeti, sugar, mandondo, misiska and pinto beans will provide an average of 56.47, 56.77, 57.91, 58.35, 64.97 % DV of protein and 177.29, 207.89, 235.08, 243.89, 214.45 % DV of fiber, respectively. Thus, consumers will require reduced serving portions of sprouted common beans to meet their daily bodily needs of protein and fiber, without exceeding energy intakes, compared to their raw forms.

Significantly, findings from this study can be correlated with dietary interventions to curtail kwashiorkor among children in developing countries. Additionally, sprouted common beans reported from this study can serve as potential nutrient-dense feeding materials for plant-based food industries involved in the production of natural minimally processed foods (e.g., meat analogs, milk, breakfast cereals, baked goods etc.), that have low energy density.



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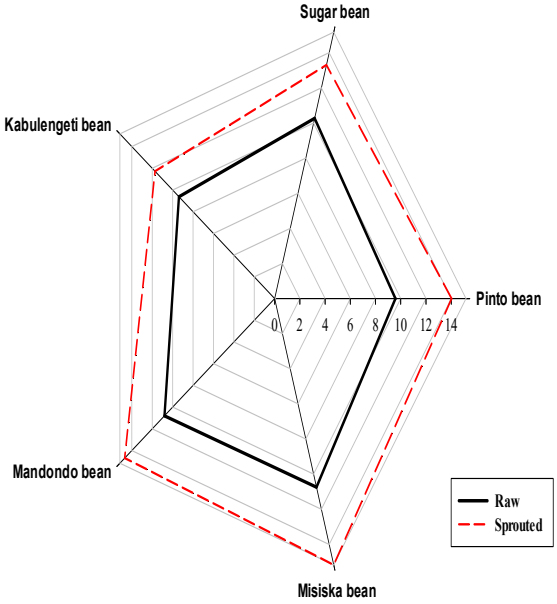

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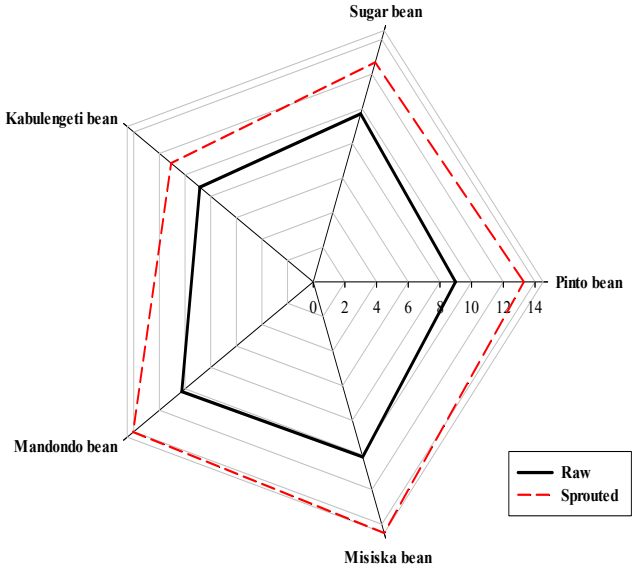


c.

Figure 3.2 a-c: Radar chart showing the mean values for Nutrient Rich Food Index (NRF9.3), a, Ratio of Recommended to Restricted Nutrients (RRR), b, index and SAIN LIM, c, index of 5 cultivars of raw and sprouted common bean

3.4.5 Nutraceutical composition

3.4.5.1 Phenolic compounds and total phenolic compound index

Overall, sprouting effected significant ($P < 0.05$) increments in phenolic compounds for all bean sprouts, as depicted in Table 3.3. After sprouting, total phenolic acids increased by 516, 1320, 378, 1145 and 2600 % for sprouts of pinto, sugar, kabulengeti, mandondo and misiska, respectively. Total flavonoids after sprouting also increased by 5624, 1835, 1989, 595, and 538 %, for sprouts of pinto, sugar, kabulengeti, mandondo, and misiska, respectively. Furthermore, tartaric acids also increased significantly by 946, 143, 321, 158, and 231 % for sprouted pinto, sugar, kabulengeti, mandondo, and misiska, respectively, with anthocyanins also increasing by 61.82, 21.79, 24.41, 26.15, and 39.04 %.

According to Mendoza-Sánchez et al. (2016) sprouting initiates diverse bio-chemical reactions that can induce the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). For sprouts to protect themselves from these oxidative species, there is the signaling of endogenous defense genes responsible for triggering the induction of plant defense enzymes such as phenylalanine ammonia-lyase (i.e. the main enzyme responsible for biosynthesis of phenolic compounds by initiating the phenylpropanoid pathway). Thus, our observed increases of phenolic compounds may be due to the triggering of the shikimate and phenylpropanoid pathways, responsible for biosynthesis of phenolic compounds. Also, activities of cellulases and pectinases during sprouting may break down ester linkages that bind proteins and cell wall matrixes to bound phenolic compounds, thus liberating bound phenolics into cell vacuoles and increasing their bio-accessibility (Gan et al., 2017).

Furthermore, total polyphenol index (TPI) of sprouted beans were significantly higher compared to raw beans (Fig 3.3). Whereas raw beans reported TPI of 1.00, sprouting caused the

highest PI of 3.09 in sugar beans, followed by pinto (2.61), kabulengeti (2.13), misiska (2.08) and mandondo (1.72). Increased TPI values observed for sprouted beans indicate their overall richness in different polyphenolic compounds, thus, granting beans sprouts a nutraceutical synergy, compared to their raw counterparts. In literature, polyphenolic compounds are reported to offer a duo protection against cell oxidative stress by scavenging generated free radicals and also preventing the initiation of oxidative stress in healthy cells. Thus, the inclusion of polyphenolic rich beans sprouts reported from this study in diets and minimally processed foods, will help protect consumers against risks of cancers, cardiovascular diseases, hypertension, diabetes and other chronic diseases. With the current consumer crave for natural nutraceutical foods, the food industry can take advantage of sprouting as a minimal processing approach to provide consumers with beans sprouts that are phenolic-rich, ready-to-eat or milled to flour for diverse use.

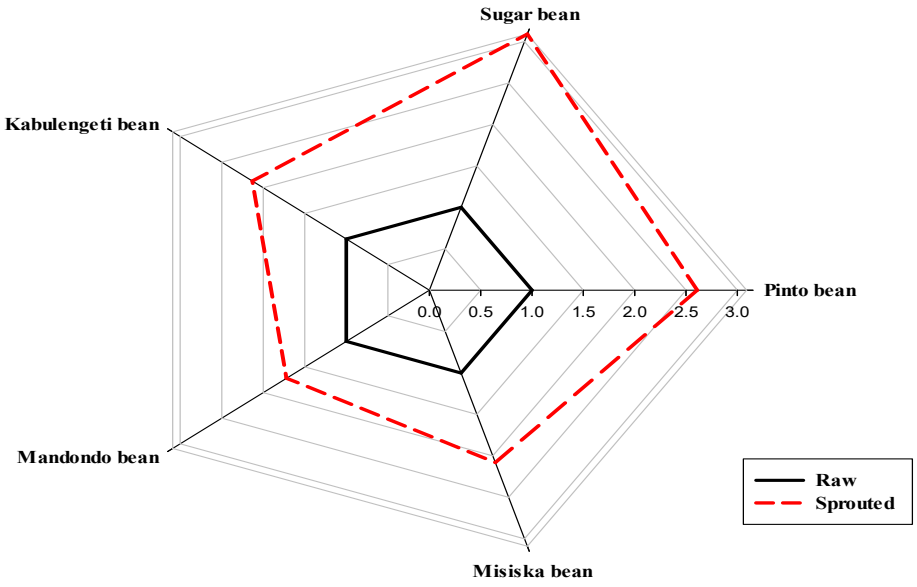


Figure 3.3: Radar chart showing mean values for total phenolic index of 5 cultivars of raw and sprouted common bean. Data represent mean values ± SD of triplicates

Table 3.3: Composition of phenolic compounds in raw and sprouted common bean cultivars (mg/100 g, dry weight basis)

Cultivar	Total phenolic acids	Tartaric esters	Anthocyanins	Total flavonoids
Raw				
PBR[1]	5.10±0.00^{f}	0.68±0.02^{e}	1.64±0.04^{g}	2.13±0.17^{g}
SBR	4.82±2.27^{f}	0.69±0.01^{e}	1.68±0.02^{fg}	2.42±0.10^{g}
KBR	3.76±1.53^{f}	0.40±0.01^{f}	1.64±0.04^{g}	2.24±0.18^{g}
MDR	3.39±2.30^{f}	2.17±0.03^{c}	1.76±0.00^{e}	8.24±0.13^{f}
MSR	2.06±0.04^{f}	1.69±0.04^{d}	1.73±0.00^{ef}	9.37±0.12^{e}
Total	19.1293	5.6350	8.4594	24.4007
Sprouted				
PBS	31.47±0.49^{d}	7.09±0.00^{a}	2.66±0.02^{a}	58.37±0.02^{b}
SBS	68.48±1.40^{a}	1.69±0.00^{d}	2.05±0.00^{d}	46.80±0.04^{d}
KBS	18.00±3.81^{e}	1.69±0.00^{d}	2.05±0.00^{d}	46.81±0.05^{d}
MDS	42.22±6.10^{c}	5.59±0.00^{b}	2.22±0.00^{c}	57.33±0.12^{c}
MSS	55.53±0.02^{b}	5.59±0.00^{b}	2.40±0.00^{b}	59.84±0.00^{a}
Total	215.7003	21.6528	11.3791	269.1599

Mean values with different superscripts in a column for a bean type (Raw, R or Sprouted, S) are significantly different from each other when $P < 0.05$. [1]PB – Pinto bean; SB – Sugar bean; KB – Kabulengeti bean; MD – Mandondo bean; MS – Misiska bean

3.4.5.2 Antioxidant activities and total antioxidant index

Overall, phenolic extracts from all sprouted beans showed significantly ($P < 0.05$) higher inhibition of DPPH free radicals compared to their raw bean counterparts, as shown in Figure 3.4a. We observed increasing antioxidant activity at increasing phenolic extract concentrations (250-550 µg/mL). Scavenging of DPPH free radicals ranged between 25.31-75.27 and 39.29-84.82 % among raw and sprouted beans cultivars, respectively. In increasing order of phenolic extract concentrations, scavenging of DPPH free radicals increased from 50.40 to 55.36, 54.50 to 62.80, 75.27 to 79.46, 58.04 to 81.84, and 66.50 to 84.82 %, for kabulengeti, sugar, mandondo, pinto and misiska beans, respectively, after sprouting. Findings of the present work are consistent with the work of Fouad and Rehab (2015) who reported on DPPH free radical scavenging

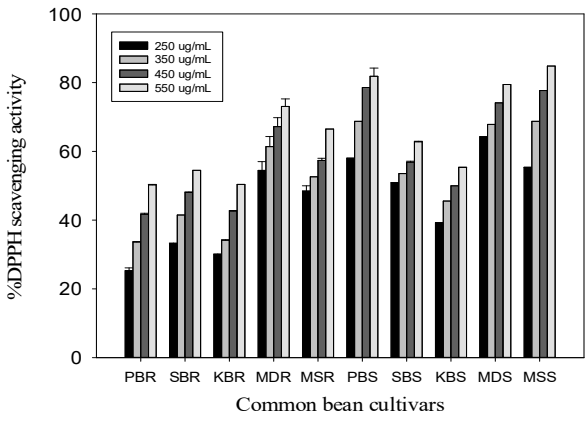
activity for sprouted and raw lentil seeds as 62.19 and 40.76%, respectively. Enhanced antioxidant capacity in beans sprouts can be attributed to their reported higher concentrations of polyphenolic compounds (Table 3.4), which can chelate free radicals due to their structural make-up of hydroxyl groups. In scavenging DPPH free radicals, reported phenolic compounds in beans were able to donate hydrogen atoms or electrons to generated DPPH free radicals, thus decreasing their stability and subsequent quenching.

As shown in Fig. 3.4b, although sprouted beans showed higher ABTS scavenging activity than their raw forms, this trend was more pronounced in cultivars with high phenolic contents (i.e., mandondo and misiska beans sprouts). Variations among capacities of phenolic extracts from different beans cultivars to scavenge ABTS free radicals may be due to the number of hydrogen atoms being donated by phenolic compounds that comprises each extract. Results observed from ABTS scavenging assay were consistent with our observations from DPPH assay. However, percentage scavenging capacity reported for ABTS assay, were higher compared to DPPH assay.

According to Kim et al. (2002) ABTS assay is applicable to both hydrophilic and lipophilic antioxidant reaction systems, whereas DPPH assay is favorable to hydrophobic antioxidant reaction systems. Thus, polarities of extract phenolic compounds and their related affinities to ABTS and DPPH reaction mediums may have contributed to variations in our reported antioxidant assays. Excess ferrous iron in biological systems can trigger the induction of free radical reactions, by donating electrons, even to non-reactive radicals. Overall, chelating capacity for raw and sprouted beans ranged between 22.70-80.87 and 40.67-97.11 %, respectively. Fe^{2+} chelation capacity for raw versus sprouted beans showed significant increments in a concentration dependent trend, except for sprouts of misiska and mandondo,

statistically. Ferrous iron reductions by phenolic extracts from this study may be attributed to effective electron-donating abilities of phenolic compounds present in extracts (Roginsky and Lissi 2005).

Antioxidant capacity of a food system is contributed by diverse mechanisms, thus making it difficult to quantitatively define total antioxidant capacity of a food by a simple method. Applying the proposed total antioxidant activity index (AI) of Swieca et al. (2014), we observed elevated AI for all sprouted beans, compared to raw beans (Fig. 3.5). Compared to raw beans (1.00), the highest value of AI was observed for sprouted pinto (1.76), followed by kabulengeti (1.28), misiska (1.28), sugar (1.26) and mandondo (1.10) beans. Although sprouted sugar beans reported the highest PI value of 3.09, it didn't show the highest AI value of 1.76, signifying that, AI capacity of a food does not only depend on polyphenol concentrations but also on the chemical nature, and stability of polyphenols during antioxidant mechanisms. Elevated AI of bean sprouts denotes their potential as natural dietary nutraceutical agents against oxidative stress and its negative risks of chronic diseases and various cancers. Thus, sprouting when performed under controlled conditions, can be a sustainable process for improving the nutraceutical quality of common beans.



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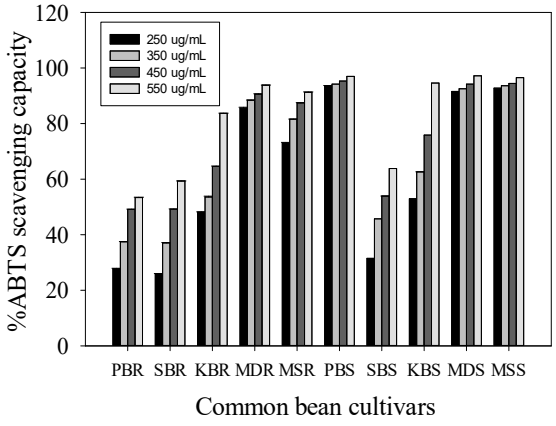

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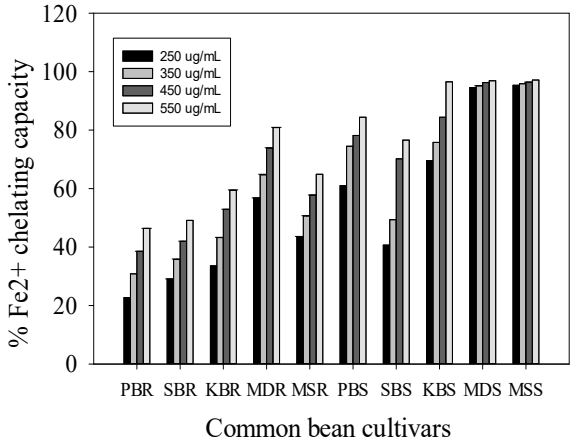


c.

Figure 3.4 a-c: Charts showing the mean values for DPPH, a, ABTS, b, and Fe^{2+}, c, scavenging activities of 5 cultivars of raw (R) and sprouted (S) common beans at different concentrations of phenolic extracts. Data represent mean values ± SE of triplicates. PB - Pinto bean; SB - Sugar bean; KB - Kabulengeti bean; MD - Mandondo bean; MS – Misiska bean

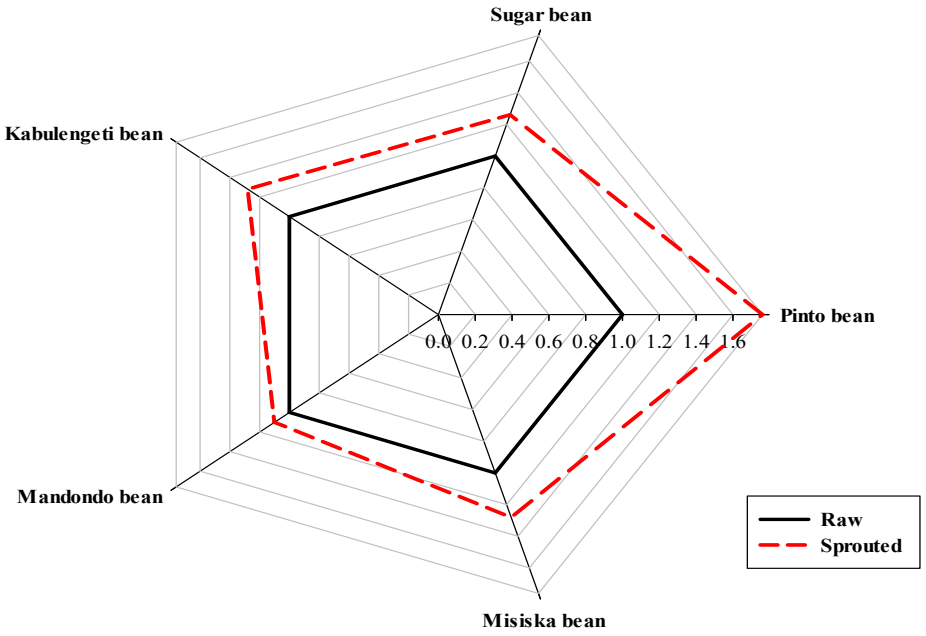


Figure 3.5: Radar chart showing mean values for total antioxidant index of 5 cultivars of raw and sprouted common beans. Data represent mean values ± SD of triplicate

3.5 Conclusion

The results of this study show that, application of sprouting was an effective process for improvement of nutritional value of common beans sprouts by increasing nutrient-density and reducing total antinutritional index. Also, from this study, nutraceutical benefits of sprouted beans were improved by an increase in phenolic compounds and total phenolic index, resulting in subsequent elevated antioxidant potential. Thus, sprouted beans with improved nutrient density and bioactive compounds can be consumed directly as ready-to-eat foods or formulated into food products as dietary interventions against malnutrition and development of chronic diseases. Theoretically, sprouting enhanced nutraceutical quality of common beans sprouts, thus, can be exploited as a simple tool for providing consumers with bioactive low-processed foods, such as edible beans sprouts.

CONNECTING STATEMENT TO CHAPTER IV

The capacity of sprouting to enhance nutrient-density, phenolic accumulation and antioxidative properties of common bean was established in chapter III of this thesis. With the current consumer demand for natural phenolic-dense foods, sprouted common bean can be used as natural minimally processed vegetables or dehydrated for use in food formulations. However, results of chapter III showed significant variations in phenolic contents of sprouts depending on common bean cultivar. Thus, the objective of chapter IV was to increase the rate of phenolic synthesis at different sprouting stages of common bean by chemical elicitation. Common bean sprouts were treated with concentrations of glutamic acid and NaCl elicitors and the effect on stimulation of stress markers, phenylpropanoid triggering enzymes, phenolic accumulations, antioxidative and sprout morphological properties were studied. This chapter has been prepared for submission in the Journal of Food Science and Nutrition as follows:

Ampofo, J. O. and Ngadi, M. (2020). Phenolic biosynthesis and antioxidant properties of common bean (*Phaseolus vulgaris*) sprouts under glutamic acid and sodium chloride elicitations. To be submitted to Journal of Food Science and Nutrition.

CHAPTER IV

Phenolic biosynthesis and antioxidant properties of common bean (*Phaseolus vulgaris*) sprouts under glutamic acid and sodium chloride elicitations

4.1 Abstract:

This study evaluated the impact of different concentrations of glutamic acid (1, 3, 5 and 7 mM) and sodium chloride (NaCl- 100, 200, 300 and 400 mM) elicitations on biosynthesis of phenolic compounds and antioxidant properties of common bean sprouts. Results showed that high glutamic acid concentrations (5 and 7 mM) elicited maximum levels of stress markers (H_2O_2, catalase and peroxidase), defense phenolic triggering enzymes (phenylalanine ammonia-lyase; PAL and tyrosine ammonia-lyase; TAL), and subsequent elevation of phenolic compounds. Although stress markers were elevated, it recorded no negative influence on the sprouting potential of common beans. Maximum antioxidant capacities were elicited with 5 mM glutamic acid after 96 hr of sprouting, over the NaCl and control treatments. Multiple quadratic regression studies led to the conclusion that, for optimum biosynthesis of phenolic compounds, synergistic activities of PAL and TAL are required rather than in their isolated catalytic activities.

4.2 Introduction

Phenolic compounds are plant secondary metabolites grouped according to their chemical structure into flavonoids (e.g., flavones, flavonols, isoflavones etc.), phenolic acids (e.g., hydroxybenzoic and hydroxycinnamic acid derivatives) and proanthocyanins (Liu et al., 2019). Notable examples of phenolic-rich foods include fruits (e.g., grapes, blueberries etc.,), vegetables (e.g., carrots, green leafy vegetables etc.,), pulses (e.g., lentils, common beans, chickpeas etc.,) etc. In plants, phenolic compounds act as defensive agents against environmental stress and predators, as well as helping to attract pollinating agents. Literature has documented vast research on positive health benefits of phenolic compounds including antioxidant, antidiabetic, anti-tumor, anti-inflammation, and anti-allergenic activities (Guo et al., 2012).

However, common beans have varied concentrations of phenolic compounds depending on variety and growing conditions. For instance, Telles, Kupski and Furlong (2017) studied total phenolic acids contents of red, carioca, white and black beans, and reported their concentrations as 1.8, 1.2, 0.19 and 0.49 mg GAE/g, respectively. In another study by Xu, Yuan, and Chang (2007) total flavonoids levels for navy, red kidney, pinto and pink beans were recorded to be 0.92, 3.39, 2.99 and 3.65 mg CE/g, respectively. Thus, due to these variations, there is the need to develop methods that could increase contents of phenolic compounds in common beans, especially in varieties with minimal levels. Sprouting, an early developmental growth phase in plants has been reported as a potential tool for enhancing phenolic levels in common beans (Ampofo and Ngadi, 2020; Xue et al., 2016; Aguilera et al., 2014). Because phenolic compounds are bio-synthesized under environmental stress, it is anticipated that environmental factors involved in sprouting may be manipulated to further enhance the accumulation of phenolic compounds.

A potential sustainable approach for this purpose is the mechanism of elicitation. Plant elicitation involves the manipulation of physiological processes towards an increased triggering of secondary metabolites through the use of elicitors/stressors. An elicitor/stressor can be biotic or abiotic, with biotic elicitors being obtained from a biological origin (e.g., proteins, phytohormones etc.,) whereas abiotic types are obtained from non-biological origins (e.g., salts and physical factors) as reviewed by Owolabi, Yupanqui and Siripongyutikron (2018). Different forms of biotic (e.g., lactoferrin, glutamic acid, chitosan) and abiotic (e.g., light, NaCl, hypoxia) elicitors/stressors have been reported to enhance levels of phenolic compounds in common bean sprouts. Physiological changes induced by elicitation leads to the accumulation of reactive oxygen species (e.g., peroxides, singlet oxygen, etc.) at elevated levels. Accordingly, defense pathways (pentose phosphate, shikimate and phenylpropanoid pathways) are triggered to induce the expression of phenylpropanoid triggering enzymes (phenylalanine ammonia-lyase and tyrosine ammonia-lyase) at maximum levels, resulting in increase of phenolic compounds (Mendoza-Sanchez et al., 2016). Limón et al. (2014) studied the effect of 5 mM glutamic acid elicitation on accumulation of soluble phenolic compounds of kidney bean sprouts, reporting a 13.86% significant increase after 8 days of elicitation compared to the control sprouts. Similarly, Swieca, Seczyk and Gawlik-Dziki (2014), and Mendoza-Sanchez et al. (2016) observed significant increases in phenolic compounds with NaCl elicited lentil and salicylic acid elicited common beans sprouts, respectively, compared to their control counterparts.

However, there is currently lack of knowledge on the relations between elicitor levels, accumulation of stress markers, stimulation of phenylpropanoid triggering enzymes and production of phenolic compounds in common bean sprouts. This knowledge will be crucial in designing processing systems for the production of phenolic-enriched sprouted beans. Thus, this

study was set to provide a deeper understanding of chemical elicitation in the production of phenolic-rich sprouts. In this study, effects of glutamic acid and NaCl elicitors on biosynthesis of phenolic compounds in common bean sprouts were evaluated. Glutamic acid and NaCl were chosen because literature have linked glutamic acid and NaCl to accumulation of phenolic compounds through proline biosynthesis and osmotic stress, respectively. The specific objectives of the study were to evaluate the effects of different concentrations of glutamic acid and NaCl on morphological characteristics, stress markers, defense phenylpropanoid triggering enzymes, accumulation of phenolic compoundsa and antioxidant capacities at different sprouting stages of common bean.

4.3 Materials and methods

4.3.1 Common bean seeds

Common bean cultivar 'Kabulengeti' was provided by the Council for Scientific and Industrial Research Center of Zambia. This cultivar has high yield stability; high tolerance to diseases and pests; comprises of protein and lipid contents of 24.38 and 1.38 %, respectively; the most widely consumed variety in Zambia and most of East Africa. However, it has limited concentrations of phenolic acids (3.76 mg CE/100 g) and flavonoids (2.24 mg RE/100 g), thus, it was selected for this study as a model for phenolic limiting common bean cultivars.

4.3.2 Chemical elicitors and their preparation

Glutamic acid and NaCl were obtained from Sigma-Aldrich, Oakville-Ontario, Canada. The elicitors were dissolved in distilled water at the following concentrations: Glutamic acid (1, 3, 5

and 7 mM) and NaCl (100, 200, 300 and 400 mM). All elicitor solutions were freshly prepared at each day of application.

4.3.3 Sprouting elicitation process

Bean seeds (100 g) were disinfected with 1% sodium hypochlorite (500 mL) for 20 min and rinsed with distilled water until neutral pH was attained. Next, the seeds were soaked in distilled water (1:3 w/v) at 25 ºC for 24 h. Hydrated seeds were sprouted in an incubator at 25 ºC in darkness for 24, 48, 72 and 96 h on petri dishes lined with absorbent paper. During sprouting, glutamic and NaCl elicitations were carried out by spraying the sprouts with 25 mL of each elicitor solution at intervals of 6 h. Control sprouts were hydrated with distilled water instead of elicitor solutions. After each sprouting time, obtained sprouts were measured, frozen, lyophilized, and milled with a Cyclone Sample Mill (UDY Corp., Fort Collins, CO, USA) fitted with a 1 mm screen for analysis.

4.3.4 Evaluation of morphological characteristics

4.3.4.1 Physical parameters

In order to determine how elicitation influenced physical properties of sprouts, radicle length (cm) and hypocotyl diameter (mm) of sprouts were measured at 12 h interval from the onset of sprouting, by using a ruler and Vernier caliper, respectively.

4.3.4.2 Sprouting potential

Sprout vigor index, sprouting index and sprouting percentage were used to describe sprouting capacity after every 12 h of treatment, by using the following formulas (Bormashenko et al., 2012; Ling et al., 2014):

Sprouting percentage (%): $S_p = N_t/N_{TS}$ Eq. (4.1)

Sprouting index: $S_i = \sum N_t/t$ Eq. (4.2)

Sprout vigor index: $S_{vi} = S_i \times L$ Eq. (4.3)

where S_p, S_i *and* S_{vi} are sprouting percentage, sprouting index and sprout vigor index, respectively. N_t is the number of total sprouts showing emerged radicle at time t; N_{TS} is the total number of seeds on the petri dish; *t* is the sprouting time *and L* is the total length of sprout.

4.3.5 Determination of stress markers

4.3.5.1 Hydrogen peroxide analysis

Hydrogen peroxide (H_2O_2) levels were evaluated as described by Alexieva et al. (2001). Briefly, 0.1 g of sample was homogenized with 1 mL of a solution containing 0.25 mL trichloroacetic acid (TCA) (0.1%, w:v), 0.5 mL KI (1 M) and 0.25 mL potassium phosphate buffer (10 mM, pH 7.0) at 4 °C for 10 min. Afterwards, the obtained mixture was centrifuged (10000 g, 15 min, 4 °C), supernatant kept in the dark for 30 min and absorbance recorded at 390 nm with a microplate reader. A control was prepared by replacing sample with 0.1% TCA in the reaction mixture.

4.3.5.2 Stress indicative enzymes

4.3.5.2.1 Extract preparations and protein content determination

4.3.5.2.2 Total protein assay

Protein contents of enzyme extracts were measured according to the method of Bradford (1976). Bradford dye reagent was prepared by diluting the commercial dye concentrate in a 1:4 ratio with distilled water. To the 20 µL enzyme extract, 1 mL of the prepared Bradford dye reagent was added and incubated at room temperature for 5 min. Afterwards, the reaction mixture was vortexed for 5 sec, and absorbances read at 595 nm using a microplate reader. Blanks were prepared under the same conditions, by replacing sample enzyme extracts with extraction buffer.

4.3.5.2.3 Enzyme extraction

Extracts of catalase (CAT) and peroxidase (POD) were performed as described by Swieca, Seczyk and Gawlik-Dziki (2014). Briefly, 200 mg of sample flour was homogenized with 4 mL of 100 mM sodium phosphate buffer (pH 6.4) containing 0.2 g of polyvinylpyrrolidone, at 4 °C for 20 min. Afterwards, homogenized mixture was centrifuged (12000 g, 4 °C, 30 min), and the supernatant was collected as enzyme extract. Enzymatic and protein assays were performed on the same day of extraction.

4.3.5.2.4 Enzymatic assays

To evaluate catalase (CAT) activity, a reaction mixture comprising of 0.05 mL of enzyme extract and 0.95 mL of H_2O_2 (10 mM, in 100 mM sodium phosphate buffer, pH 7) was prepared. Decomposition of H_2O_2 was monitored by a decrease in absorbance at 240 nm after 1 min at 30

°C. One unit was defined as the amount of CAT that decomposed 1.0 μmol H_2O_2 per min under the conditions of the assay. Results were expressed as U/mg of protein (Swieca, Seczyk and Gawlik-Dziki, 2014).

For peroxidase (POD) activity, the reaction mixture consisted of 0.1 mL of enzyme extract and 2 mL of guaiacol (8 mM, in 100 mM sodium phosphate buffer, pH 6.4) incubated for 1 min at 30 °C. Afterwards, 1 mL of H_2O_2 (24 mM) was added to the reaction mixture and the increase in absorbance was measured at 460 nm using a microplate reader. POD activity was expressed as U, where 1 U = 0.001 Δ460/min under the conditions of the assay. Results were presented as U/mg of protein (Swieca, Seczyk and Gawlik-Dziki, 2014).

4.3.6 Evaluation of the defense phenylpropanoid triggering enzymes

4.3.6.1 Enzyme extraction

Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) extractions were performed at 4 °C, as outlined by Swieca, Seczyk and Gawlik-Dziki (2014). Two hundred (200) mg of sample flour was homogenized with 2 mL extracting buffer (0.2 M boric acid buffer containing, 1 mM EDTA and 50 mM β-mercaptoethanol, pH 8.8) for 20 min. The homogenized mixture was centrifuged (12000 g, 30 min) and the supernatant was collected as enzyme extracts.

4.3.6.2 Enzymatic assays

PAL assay was evaluated by incubating a reaction mixture comprising of 300 μL of enzyme extract, 1.2 mL 0.02 M L-phenylalanine and 2 mL of the PAL extracting buffer at 30 °C for 60 min. After incubation, 0.5 mL of 10% TCA was added to stop the reaction, and centrifuged (15000 g, 10 min). Absorbance of supernatant was measured at 290 nm by using a

microplate reader. One unit was defined as the amount of enzyme that produced 1.0 µmol trans-cinnamic acid per min under conditions of the assay. Results were expressed as U/mg of protein (Assis et al., 2001).

For TAL assay, 100 µL of enzyme extract was incubated with 0.9 mL of 0.02 M L-tyrosine at 30 °C for 60 min. After incubations, 0.5 mL of 10% TCA was added to stop the reaction, and the mixture was centrifuged (15000 g, 10 min). Absorbance of supernatant was measured at 310 nm using a microplate reader. One unit was defined as the amount of enzyme that produced 1.0 µmol *p*-coumaric acid per min under the conditions of the assay. Results were calculated as U/mg of protein (Assis et al., 2001).

4.3.7 Determination of phenolic compounds and antioxidative properties

4.3.7.1 Extraction of phenolic compounds

Phenolic compounds were extracted as described by Marathe et al. (2011). About 1 g of sample flour was extracted with 15 mL of 80% aqueous methanol (V/V), centrifuged (5000 rpm, 10 min), and supernatant filtered. Afterwards, methanol in supernatant was evaporated using a rotary evaporator, and obtained phenolic extracts were freeze-dried for subsequent analysis.

4.3.7.2 Phenolic constituents

Phenolic freeze-dried extracts were dissolved in 80% methanol and filtered through 0.45 µm cellulose acetate filter (Millipore) prior to analysis. Exactly 20 µL of the dissolved sample phenolic compound extract was mixed with 240 µL of 2% HCL in 75% methanol, vortexed for 1 min and absorbances read with microplate reader at 280 and 520 nm for total phenolic acids and anthocyanins, respectively. Standard curves were prepared under similar conditions with (+)-

catechin (0-200 µgmL^{-1}) and cyanidin-3-glucoside (0-20 µgmL^{-1}) for quantitative measurements of total phenolic acids and anthocyanins, respectively. Total phenolic acids and anthocyanins were calculated as catechin and cyanidin-3-glucoside equivalents in mg/100 g of sample flour, respectively, (Mazza et al., 1999).

Total flavonoid was measured according to the method of Hairi, Sallé and Andary (1991). Phenolic freeze-dried extracts were dissolved in 80% methanol and filtered through a 0.45 µm cellulose acetate filter (Millipore) prior to analysis. Exactly 50 µL of dissolved sample phenolic compound extract was mixed with 200 µL of 10 gL^{-1} 2-aminoethyldiphenylborate, vortexed for 1 min, and absorbance measured with microplate reader at 404 nm. A standard curve for quantitative measurement was prepared under the same condition using rutin (0-50 µgmL^{-1}). Total flavonoid content was calculated as rutin equivalents in mg/100 g of sample flour.

4.3.7.3 Antioxidant activities

4.3.7.3.1 Estimation of DPPH free radical scavenging activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging capacity was measured according to the method of Akond et al. (2011). Briefly, 83 µL of phenolic compound extract was mixed with 167 µL of 0.1 mM DPPH solution, vortexed for 1 min, kept in the dark for 30 min, and absorbance measured with microplate reader at 517 nm. A control reaction mixture was prepared by replacing phenolic compound extract with 80% methanol under the same conditions. Percentage antioxidant activity was calculated using the equation below:

$$\% \text{ Scavenging activity} = \frac{A_{control} - A_{test}}{A_{control}} \times 100 \qquad \text{Eq. (4.4)}$$

where $A_{control}$ and A_{test} are the absorbance of control and sample extract, respectively.

4.3.7.3.2 Estimation of ABTS scavenging activity

ABTS scavenging capacity was determined as described by Re et al. (1999). ABTS free radical solution was prepared by adding 5 mL of 14 mM ABTS solution to 5 mL of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution and stored in the dark for 16 h at 25 °C. Prior to using this solution, it was diluted with distilled water to get an absorbance of 0.900 ± 0.020 at 734 nm. Final reaction mixture comprised of 200 µL ABTS free radical solution and 50 µL sample phenolic compound extract, vortexed for 1 min, kept in the dark for 6 min and absorbance measured with microplate reader at 734 nm. A control reaction mixture was prepared by replacing phenolic extract with 80% methanol under the same conditions. ABTS scavenging capacity was calculated using Eq. 4.4.

4.3.7.3.3 Estimation of ferrous (Fe^{2+}) chelating capacity

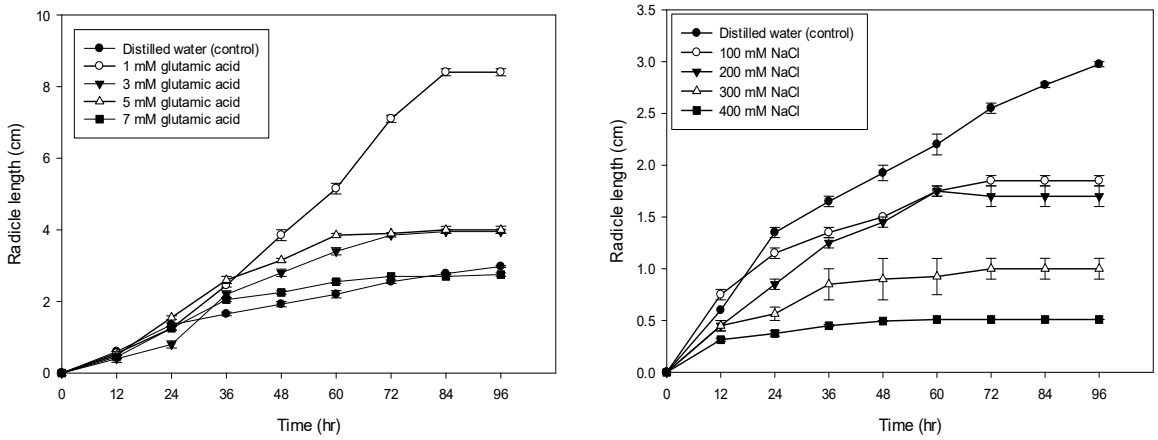
Chelation of ferrous ions by phenolic extracts was estimated by the method of Dinis, Madeira, and Almeida (1994). Briefly, 50 µL of sample phenolic compound extract was added to 0.002 M Fe (II) chloride (25 µL) and 0.005 M ferrozine (200 µL). After incubating in darkness at 25 °C for 10 min, absorbance of the mixture was measured at 562 nm with a microplate reader. Ferrous ion chelating capacity was calculated using Eq. 4.4.

4.4 Results and discussion

4.4.1 Morphological characteristics of treated sprouts

4.4.1.1 Radicle elongation and hypocotyl diameter

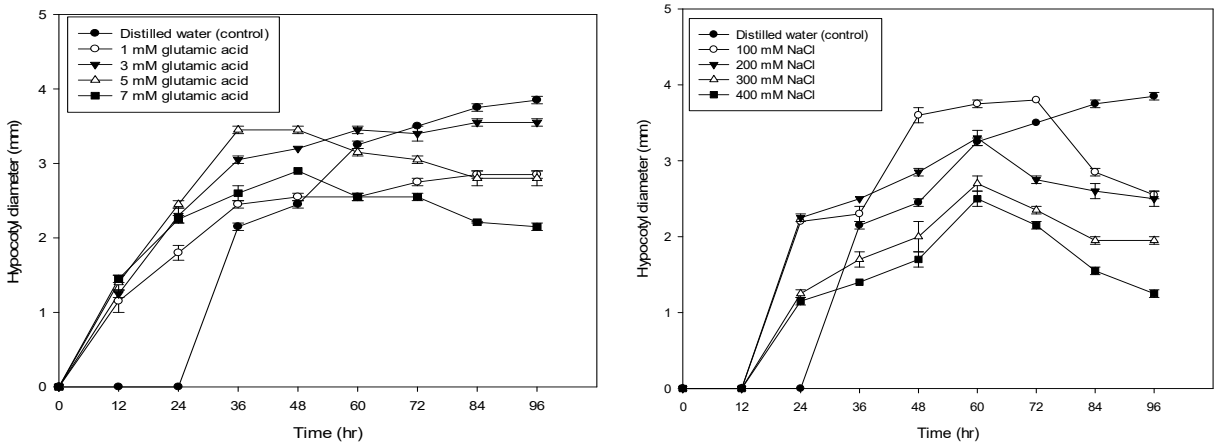
Figure 4.1a shows how glutamic acid and NaCl affected radicle growth of sprouts. Glutamic acid treatments enhanced radicle growth in comparison with NaCl and control. Amongst all the investigated treatments, 1 mM glutamic acid at 96 h sprouting significantly ($P < 0.05$) elicited the highest radicle length (64.58 % higher compared to control). This observation could be due to the increased signaling of auxin (main hormone responsible for radicle elongation) and meristematic activity at the radicle tip with low concentrations of glutamic acid, than with either higher concentrations of glutamic acid or control treatment during the sprouting process. According to Simon and Petrášek (2011) radicle elongation is regulated by two main mechanisms (a) auxin expression and distribution at radicle tip; and (b) expression of root apical meristematic cells at radicle tip. Likewise to the findings of this study, Walch-Liu et al. (2006b) observed enhanced root growth with low glutamic acid concentrations, compared to control and high glutamic acid concentration (>50 uM) treatments of *Arabidopsis thaliana* seedlings, concluding that their observation could be due to the response of root tip to changes in apoplastic glutamic acid concentration, and that some kind of signaling mechanism may be involved. Also consistent with our observation is the work of Mendoza-Sanchez et al. (2016), who observed increased radicle lengths with salicylic acid (0.1, 1 and 2 mM) and H_2O_2 (10, 20 and 30 mM) elicitations, compared to the control treatments of Dalia bean sprouts.



a. **b.**

Figure 4.1a-b: Effect of glutamic acid (a) and NaCl (b) elicitations on radicle length. Results are mean of three independent determinations ± standard error

Radicle length of sprouts obtained by control treatment was significantly higher than 100, 200, 300 and 400 mM NaCl treatments by 37.92, 42.95, 66.44 and 82.89 %, respectively, at the end of the 96 h sprouting process. Although 100 mM NaCl treatment resulted in highest radicle growth at the initial 12 h of sprouting, the trend slowed as sprouting time was prolonged. Limitations in radicle growth with NaCl may be attributed to its hyper-osmotic effects, which elevated cytoplasmic concentrations of sodium and chloride ions in treated sprouts. Hyper-osmotic conditions affect plant growth by elevating ionic strength of the cytoplasm and aleurone layer (endospermic layer responsible for synthesizing metabolic enzymes), causing reductions in cytoplasmic water, cytoplasmic streaming, substrate availability, and enzyme activities. According to Zhang et al. (2013) excess chloride ions can lead to protein and enzyme denaturation by disrupting electrostatic interactions between their amino acid blocks and final unfolding of their functional tertiary structure.



a. **b.**

Figure 4.2a-b: Effect of glutamic acid (a) and NaCl (b) elicitations on hypocotyl growth. Results are mean of three independent determinations ± standard error

As depicted in Figure 4.1b, all glutamic acid concentrations significantly ($P < 0.05$) induced early hypocotyl emergence from the seed coat and promoted hypocotyl growth within 36 h of sprouting, compared to control treatments which showed delayed hypocotyl emergence. Hypocotyl diameter continued to increase in control samples up to 96 h of sprouting. However, continued application of glutamic acid beyond 36 h and up to 96 h of sprouting, resulted in decreased hypocotyl growth as indicated by the decreased hypocotyl diameter. This observation signifies that, activity of meristematic cells localized in the hypocotyl may be negatively disrupted above certain threshold levels of glutamic acid.

Similar to glutamic acid, all NaCl concentrations induced early hypocotyl emergence compared to the control (Fig. 4.1b). Hypocotyl emerged after 12 h in glutamic acid elicited sprouts whereas it emerged after 36 h in control samples. Low NaCl concentration of 100 mM induced higher hypocotyl growth. The largest hypocotyl diameter was recorded with 100 mM at 60 h sprouting time. However, increasing NaCl concentrations up to 400 mM limited hypocotyl growth. Observed reductions with hypocotyl growth may be attributed to the negative hypertonic

effect of excess ions in the growth medium, leading to cell shrinkage and diameter reductions. According to Beffagna, Buffoni, and Busi (2005) higher concentrations of NaCl outside the plasma membrane of plant cells induces the leaching of water into the growth medium, causing the cells to shrink in size and become flaccid.

4.4.1.2 Sprouting percentage and vigor index

Elicitors significantly ($P < 0.05$) increased sprouting percentage of common beans. From Fig. 4.3a, at 24 h of sprouting all glutamic acid concentrations showed faster sprouting, with the highest effect (86 %) elicited with 5 mM glutamic acid (2.42 times significantly higher than the control at the same sprouting time). This observation may be due to the use of glutamic acid as an additional nitrogen source for efficient embryonic growth. Also, variations observed among glutamic acid concentrations could be due to differences in sensitivities of the apoplastic and symplastic regions of the cell wall and plasma membrane, respectively, to different glutamic acid concentrations. The apoplastic and symplastic regions are the respective inner layers of the cell wall and plasma membrane, responsible for regulation and transportation of gases (e.g., CO_2), solutes (e.g., Cl^-, H^+) and low molecular weight substances (e.g., hormones, amino acids etc.,) needed for physiological and metabolic processes (Pickard, 2003). Comparable to results of the current study is the work of Burguieres et al. (2007) who reported elevated sprouting percentages of green pea sprouts elicited with folic acid, than their untreated forms. However, Limon et al. (2014) observed reduced sprouting percentage with 5 mM glutamic acid elicited kidney bean sprouts, compared to their untreated forms.

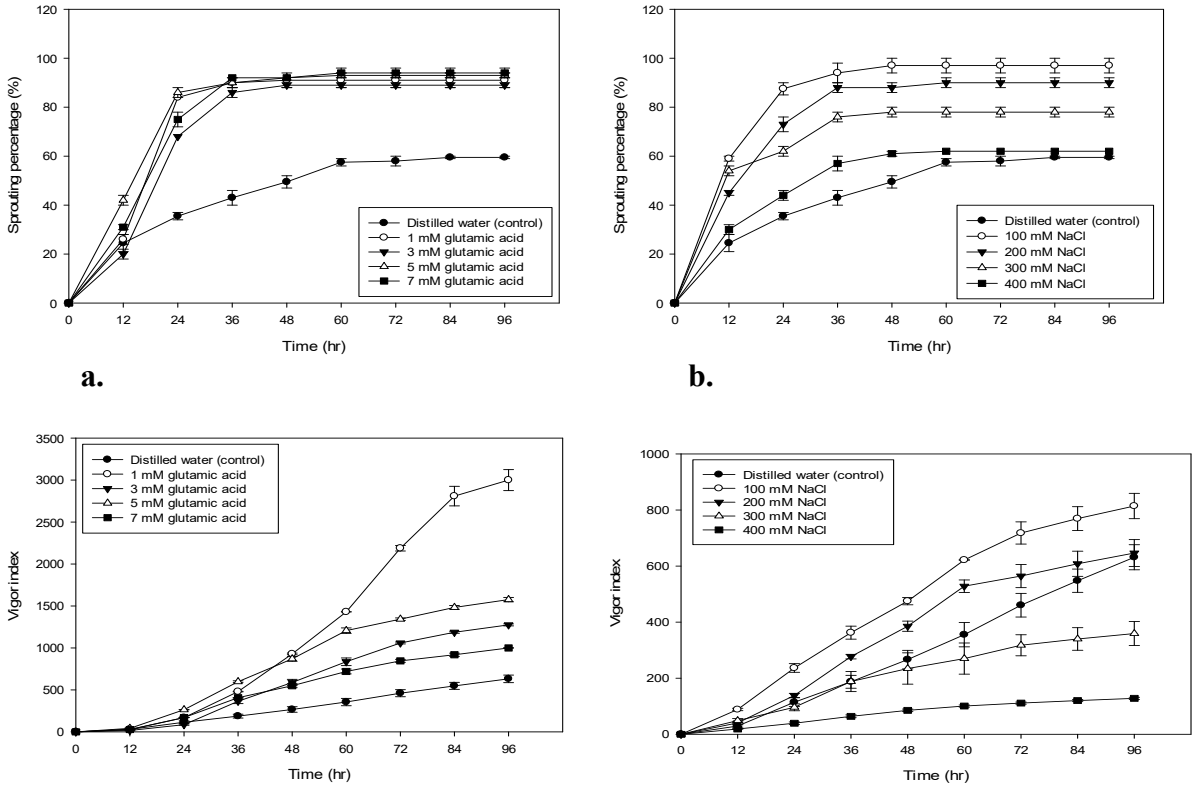


Figure 4.3(a-d): Effect of glutamic acid (a, c) and NaCl (b, d) elicitations on sprouting percentage and vigor index. Results are mean of three independent determinations ± standard error

Low concentrations of NaCl significantly increased sprouting percentage, with 100 mM NaCl eliciting the highest sprouting percentage of 88% at 24 h, compared to the control (35.50 %) (Fig. 4.3b). Although NaCl showed a stunting effect on sprout growth, it did not inhibit viability of common bean seeds to sprout. Low concentrations of NaCl have been linked to triggering of the plant hormone ethylene, which has been reported to help break seed dormancy for sprouting (Kepczynski and Kepczynska, 1997). Comparable to this study, is the work of Naseer, Nisar, and Ashraf (2001). These authors observed decreased radicle length, shoot length and sprouting percentage with increasing concentration of NaCl in barley seedlings, compared to their untreated forms, and attributed their results to ion toxicity and reduced water uptake which impeded physiological and biochemical activities.

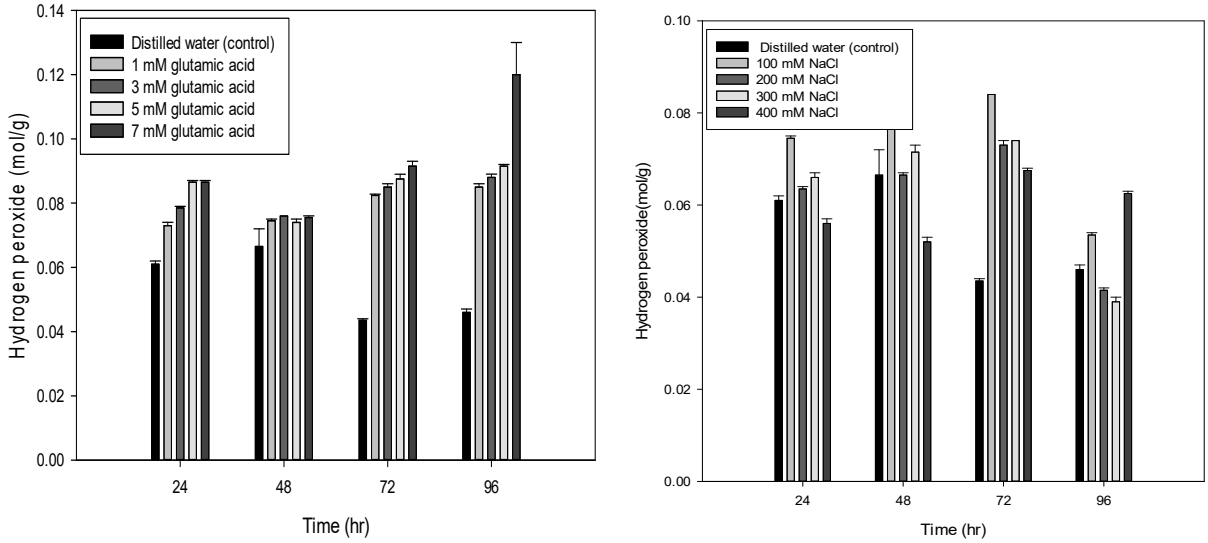
Overall, common beans from this study seem to show different sensitivities to glutamic acid and NaCl elicitations. This may be attributed to different response mechanisms of a plant system when exposed to elicitors with different biological origin, leading to the stimulation of metabolic processes at different rates. Additionally, all applied concentrations of investigated elicitors significantly ($P < 0.05$) enhanced vigor index (Fig. 4.3 c and d) over that of the control.

4.4.2 Stress markers

4.4.2.1 Accumulation of hydrogen peroxide (H_2O_2)

Phenolic compounds are secondary metabolites produced by plants in response to stress (Zhao et al., 2016). During elicitation cellular activities are increased, leading to final accumulation of reactive oxygen species (ROS) such as H_2O_2 at elevated levels (Swieca, Seczyk, and Gawlik-Dziki, 2014). Extreme levels of ROS have been reported to be deteriorative against cellular molecules, thus demanding plant defense by increasing accumulations of phenolic compounds. Therefore, in order to understand the link between stress induction and biosynthesis of phenolic compounds during elicitation of sprouts, stress markers such as H_2O_2, catalase and peroxidase were evaluated in this study.

As presented in Fig. 4.4(a-b), elicitation with glutamic acid resulted in significant ($P < 0.05$) accumulation of H_2O_2, compared to NaCl and the control. For glutamic acid elicitation, maximum level of H_2O_2 (0.12 mol/g) was elicited with 7 mM glutamic acid at 96 h of sprouting, and this result was 2.5 times higher than in the control at the same sprouting time.



a. **b.**

Figure 4.4(a-b): Effect of glutamic acid (a) and NaCl (b) elicitations on accumulation of hydrogen peroxide. Results are mean of three independent determinations ± standard error

For NaCl treatments, 100 mM NaCl elicited the highest H_2O_2 level of 0.08 mol/g (2 times higher than in the control samples) at 72 h of sprouting. It is noted that, although glutamic acid and NaCl treatments resulted in increased levels of H_2O_2, these levels were apparently non-toxic to inhibit sprouting percentage and vigor index. According to Cavusoglu and Kabar (2010) low concentrations of H_2O_2 are non-toxic, break seed dormancy, promote sprouting and seedling growth by disrupting seed hormonal balance and acting as signal molecules involved in triggering tolerance to environmental stress. Therefore, elevated H_2O_2 contents induced by the elicitors used in this study may be a possible contributory factor to their overall improved sprouting potential characteristics reported in this study.

4.4.2.2 Catalase (CAT) and peroxidase (POD) activities

Increase activities of CAT and POD have been linked to oxidative stress. The results show that elicitation with glutamic acid significantly ($P < 0.05$) elevated activities of CAT, than NaCl and the control treatments (Fig. 4.5a, b). For glutamic acid treatments, the maximum CAT activity of 23.18 U/mg protein was reported at 96 h sprouting time with the 5 mM treatment. The CAT activity value was significantly ($P < 0.05$) higher than in the control treatments by 63.19 %. CAT is an antioxidant enzyme that protects cells from oxidative damage by decomposing H_2O_2 into water and oxygen (Agrawal, Joshi, and Subramani, 2017). Thus, their elevated activities at increasing sprouting time under glutamic acid elicitations signify increments of stress and demand for sprout protection. Contrary to glutamic acid, low NaCl concentrations (100 and 200 mM) elicited high CAT activity, compared to the control and higher NaCl concentrations (300 and 400 mM). Among NaCl treatments, the maximum CAT activity was elicited with 200 mM NaCl at 96 h of sprouting and was insignificantly different compared to CAT activity with 100 mM NaCl treatment at the same sprouting time. However, this observation with 200 mM NaCl was significantly higher than in the control by 47.57 % at the same sprouting time.

For POD assay (Fig. 4.5 c and d), the greatest activity of 3.48 U/mg protein was elicited with 5 mM glutamic acid at 96 h of sprouting time and was 22.46 % significantly higher than in the control treatments and comparable to 7 mM glutamic acid treatment at the same sprouting time. This observation correlated with H_2O_2 accumulations with glutamic acid elicitation. However, NaCl elicitations significantly ($P < 0.05$) reduced POD activity compared to the control at all sprouting times. POD protect cells from oxidative stress by breaking down H_2O_2 into water and oxygen. Thus, triggered accumulations of H_2O_2 by possible elevation of metabolic processes under glutamic acid elicitations may have stimulated the increasing demand of POD

for sprout protection. Similar to our findings Randhir, Kwon, and Shetty (2009) reported maximum POD in lentil sprouts elicited with fish protein hydrolysate and oregano extract, compared to their control counterparts.

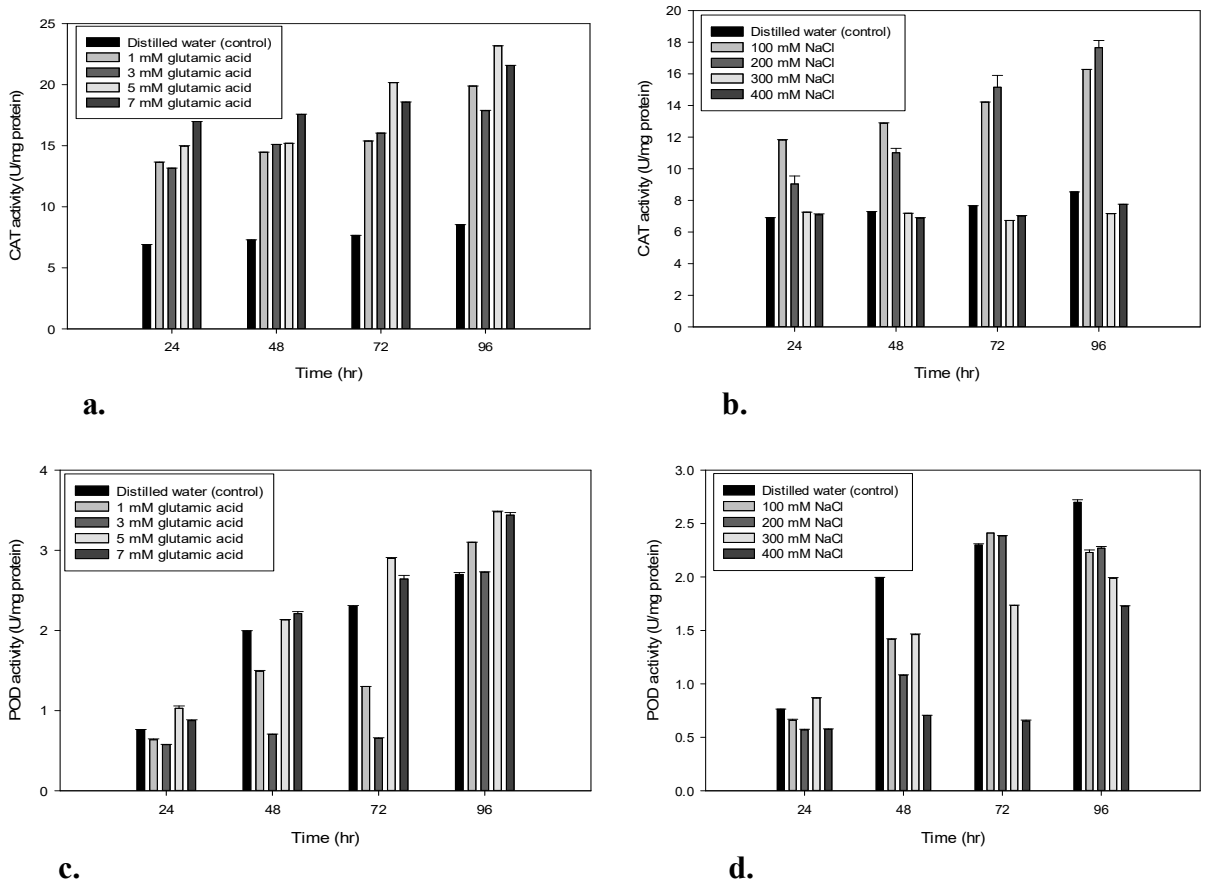


Figure 4.5(a-d): Effect of glutamic acid and NaCl elicitations on CAT (a, b) and POD (c, d) activities. Results are mean of three independent determinations ± standard error. Catalase (CAT) and Peroxidase (POD)

For the first time, monitoring of stress markers (H_2O_2, CAT and POD) during glutamic acid and NaCl elicitations of common bean sprouts have been reported by this study. It should be pointed out that response of common bean sprouts in accumulation of stress markers varied depending on elicitor nature, where glutamic acid (biotic elicitor) stimulated higher accumulations compared to NaCl (abiotic elicitor). Furthermore, although investigated stress markers increased with elicitations, this trend showed no limitations on measured characteristics

of common bean sprouting potential, compared to the control forms. Thus, suggesting that elicitation when applied under controlled conditions does not generate stress at toxic levels capable of hindering early sprout growth and development.

4.4.3 Defense phenylpropanoid triggering enzymes

4.4.3.1 Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities

According to Liu et al. (2019) stress induced during plant elicitation leads to the expression of phenylpropanoid triggering enzymes such as PAL and TAL, responsible for stimulating the biosynthesis of phenolic compounds via the phenylpropanoid pathway. In order to understand how stress markers induced by elicitors used in this study led to the biosynthesis of phenolic compounds, activities of PAL and TAL were investigated. Overall, PAL was maximally triggered with elicitations more than TAL at all sprouting times. From this study, elicitations with glutamic acid significantly ($P < 0.05$) enhanced PAL activities compared to NaCl and control treatments. As depicted in Fig. 4.6a, all glutamic acid concentrations elicited increasing PAL activity as sprouting prolonged, with the highest PAL activity of 2.57 U/mg protein elicited with 5 mM glutamic acid at 96 h of sprouting. This observation was 12.99, 24.73, 29.34 and 34.79 % significantly higher compared to 7, 1, 3 mM glutamic acid concentrations and control treatments, respectively, at the same sprouting time. For NaCl treatments, optimum PAL activity (1.77 U/mg protein) was reported for 200 mM NaCl at 72 h of sprouting, which was 10.73% higher than the control at the same sprouting time (Fig. 4.6b).

Figures 4.6c-d shows activity of TAL along the sprouting process with elicitation. As reported graphically, 5 mM glutamic acid presented the greatest TAL activity among applied glutamic acid concentrations at 96 h of sprouting, whereas lower NaCl concentrations (100 and

200 mM) elicited maximum TAL activity amongst investigated NaCl concentrations. Overall, TAL activity was increased by 31.65 and 29.16 % with 5 mM glutamic acid and 100; 200 NaCl treatments, respectively, compared to the control.

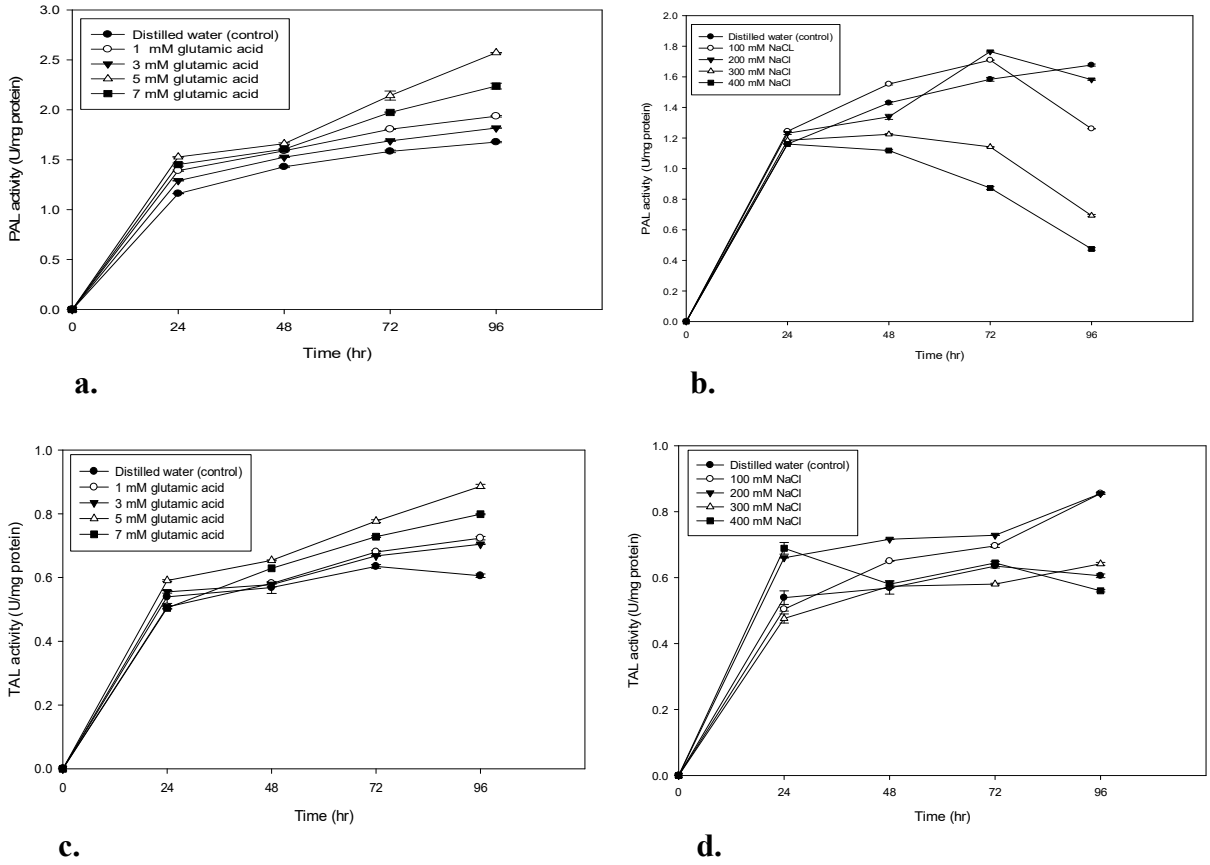


Figure 4.6(a-d): Effect of glutamic acid and NaCl elicitations on PAL (a, b) and TAL (c, d) activities. Results are mean of three independent determinations ± standard error. Phenylalanine ammonia-lyase (PAL) and Tyrosine ammonia-lyase (TAL)

Increased activities of PAL and TAL with 5 mM glutamic acid at 96 h of sprouting correlated with accumulation of stress markers (CAT and POD) at the same sprouting time. Literature has reported oxidative stress in plant species treated with elicitors, leading to the expression of genes responsible for inducing activities of defense phenylpropanoid triggering enzymes (Liu et al., 2019). Also, enhanced activities of PAL could be due to the use of glutamic acid as substrate for proline biosynthesis, a stress-antagonist amino acid in plants. Proline

biosynthesis requires NADPH as a reducing agent to convert glutamic acid into subsequent metabolites, with NADPH being a product of the pentose phosphate pathway, which itself is the source of sugar phosphate precursors required as substrates during accumulation of phenolic compounds (Burguieres et al., 2007).

Furthermore, it should also be mentioned that high concentrations of NaCl (300 and 400 mM) decreased activities of PAL and TAL, with this effect increasing drastically as sprouting was prolonged, compared to control and lower NaCl concentrations. These reductions could be due to excess chloride and sodium ions in sprout cytoplasm during elicitations, that may have altered pH and ionic concentrations, leading to negative changes in enzyme functional tertiary structure, substrate active site, availability, and final catalytic reductions (Kaya et al., 2007). For the first time, results of this study pointed out that, elicitation of common bean sprouts with 5 mM glutamic acid may better induce the phenylpropanoid pathway compared to NaCl treatments, through its maximum triggering of PAL.

4.4.4 Accumulation of phenolic compounds

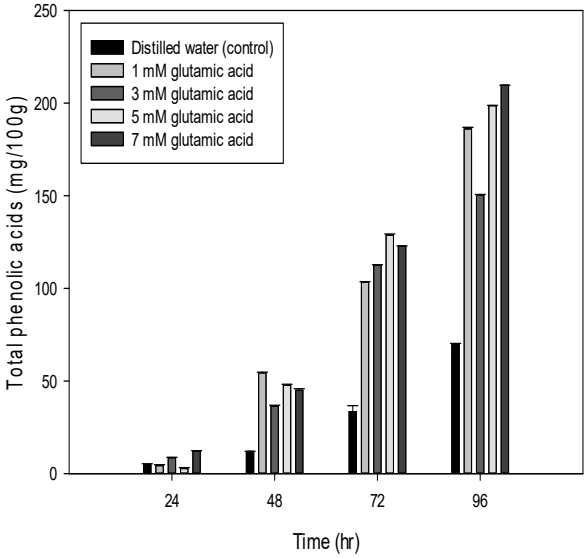
4.4.4.1 Total phenolic acids and flavonoids

Elevation of phenolic compounds in common bean sprouts was significantly attained with glutamic acid and NaCl elicitations. Among the glutamic acid treatments, the greatest level of total phenolic acids was elicited with 5 mM and 7 mM concentrations (65.00 and 66.83 %, respectively, as compared to the control treatments) at 96 h of sprouting (Fig. 4.7a). However, phenolic acids accumulated with 5 and 7 mM glutamic acid treatments were insignificant compared to each other. Also, comparing NaCl treatments to the control, 200 mM NaCl enhanced total phenolic acids by 66.83% at 72 h of spouting (Fig. 4.7b). Although 100 mM NaCl

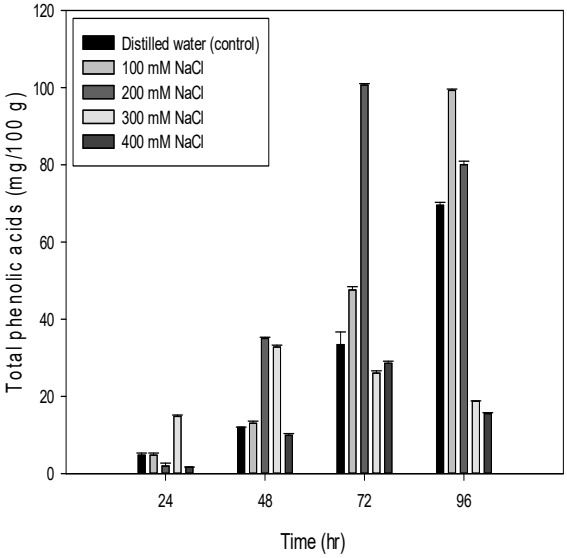
increased total phenolic acids at 96 h, this trend was insignificant compared with 200 mM effect at 72 h sprouting. Decrease in total phenolic acids observed with 200 mM at 96 h may be due to the activity of polyphenol oxidase (the enzyme responsible for browning of sprouts through the mobilization of phenols into quinones) (Cáceres et al., 2014).

Total flavonoids content was maximally elicited with glutamic acid compared to NaCl and control treatments, with this trend increasing with sprouting time. Among the glutamic acid treatments, 5 mM concentration elicited the highest total flavonoids content compared to the control at 96 h of sprouting by about 2.97 fold significant increment (Fig. 4.7c). Regarding NaCl treatments, total flavonoids was significantly enhanced with 100 mM NaCl (206.83 mg/100 g) at 96 h of sprouting, by 59.55% compared to the control at the same sprouting time (Fig. 4.7d).

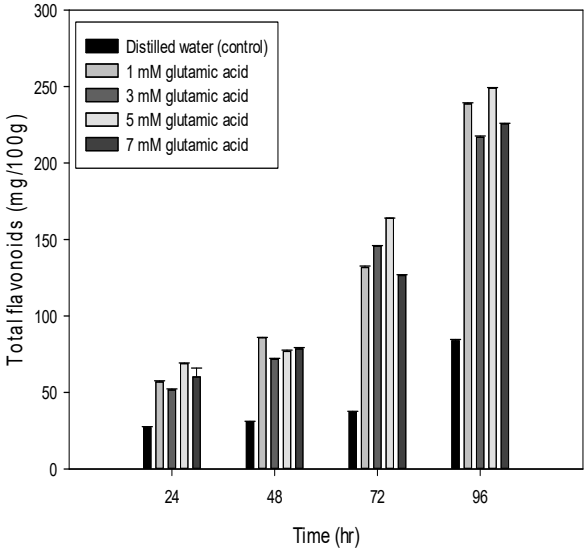
The current study showed that, glutamic acid elicitation at concentration of 5 mM enhanced levels of phenolic compounds compared to NaCl and control treatments at 96 h of sprouting. This result explains their maximum expressions of CAT, POD, PAL and TAL at the same sprouting time. Comparable to this study is the work of Mendoza-Sanchez et al. (2016). These authors investigated the effect of different concentrations of chitosan (0.7, 3.3 and 7 μM) salicylic acid (0.1, 1, and 2 mM) and H_2O_2 (10, 20 and 30 mM) on phenolic contents of Dalia bean sprouts. From their study 30 mM H_2O_2 and 2 mM salicylic acid elicited the highest levels of total phenols and flavonoids by 1.8 and 3 folds, respectively compared to their control counterparts.



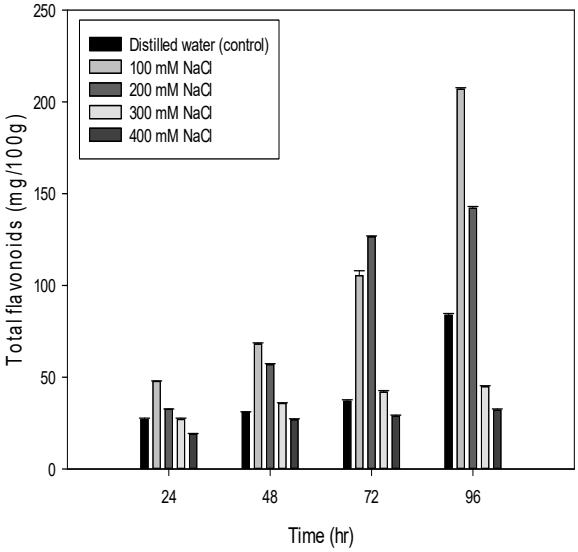
a.



b.



c.



d.

Figure 4.7(a-d): Effect of glutamic acid and NaCl elicitations on accumulation of total phenolic acids (a, b) and total flavonoids (c, d). Results are mean of three independent determinations ± standard error

4.4.5 Antioxidant capacities

From Table 4.1, ability of common bean sprouts to scavenge free radicals and chelate ferrous ions were significantly ($P < 0.05$) enhanced by elicitation, depending on elicitor type, concentration and sprouting time. Comparing glutamic acid and NaCl treatments to the control, the highest ability to scavenge DPPH free radicals was elicited with 5 mM glutamic acid at 96 h of sprouting, with this result being significantly ($P < 0.05$) higher than the control by 5.13% at the same sprouting time. Comparative to DPPH scavenging, ability to scavenge ABTS free radicals was optimum with 5 mM glutamic acid treatments by being 32.29 and 93.82 % significantly higher compared to 300 mM NaCl (reported the highest ABTS scavenging capacity among NaCl treatments) and the control at 96 h of sprouting time. Also, the greatest potential to chelate ferrous ions was obtained with 3 mM glutamic acid at 69.03%, and this result was significantly higher than with 100 mM NaCl (reported the highest ferrous chelating capacity among NaCl treatments) and the control by 15.50 and 12.75 %, respectively, at the same sprouting time.

Enhanced antioxidant capacities of phenolic extracts obtained with 5 mM glutamic acid elicitation can be linked to its high concentration of flavonoids and phenolic acids accumulated along the sprouting process compared to phenolic extracts from the control and NaCl treatments. Furthermore, variations in antioxidant capacities may be due to the types and structure (e.g., number of hydroxyl groups, degree of glycosylation etc.,) of individual phenolic compounds being elicited with glutamic acid, compared with the phenolic compounds elicited with NaCl and control treatments. According to Kim et al. (2002) phenolic compounds are able to scavenge free radicals and chelate metal ions through their hydrogen and electron donating properties, capable of reducing free radical reactivity and making them more stable. These antioxidant changes have

also been reported in broccoli sprouts elicited with 176 mM sucrose, compared to their control and was attributed to their enhanced total phenolic levels (Guo, Yuan, and Wang, 2011a).

Table 4.1: Effect of glutamic acid and NaCl elicitations on mean antioxidant activities ± SE of common bean sprouts

Effect	No. of observation (n)	Antioxidant activities (%)		
Elicitation		ABTS±SE	Fe^{2+}±SE	DPPH±SE
Distilled water (control)	8	74.39±0.31^{i}	61.11±0.31^{e}	82.74±0.11^{i}
1 mM GA	8	89.76±0.31^{b}	67.60±0.31^{c}	89.98±0.11^{d}
3 mM GA	8	89.08±0.31^{c}	69.03±0.31^{a}	90.29±0.11^{b}
5 mM GA	8	90.77±0.31^{a}	68.66±0.31^{b}	90.78±0.11^{a}
7 mM GA	8	87.47±0.31^{d}	66.03±0.31^{d}	89.84±0.11^{f}
100 mM NaCl	8	81.30±0.31^{g}	58.33±0.31^{f}	89.39±0.11^{h}
200 mM NaCl	8	84.59±0.31^{f}	56.08±0.31^{g}	89.48±0.11^{g}
300 mM NaCl	8	84.79±0.31^{e}	54.98±0.31^{h}	89.89±0.11^{e}
400 mM NaCl	8	80.08±0.31^{h}	48.06±0.31^{i}	90.27±0.11^{c}
Sprouting time (h)				
24	18	78.55±0.21^{d}	60.09±0.21^{d}	87.88±0.08^{d}
48	18	82.55±0.21^{c}	61.53±0.21^{b}	88.68±0.08^{c}
72	18	88.53±0.21^{b}	61.97±0.21^{a}	89.56±0.08^{b}
96	18	89.14±0.21^{a}	60.79±0.21^{c}	90.61±0.08^{a}

Data presented are mean ± SE of three independent observations. Mean values in a column with different superscripts are significantly different at $P < 0.05$. [1]GA - Glutamic acid; NaCl- Sodium chloride

4.4.6 Relationship between stress markers, phenylpropanoid triggering enzymes and phenolic compositions

Quadratic regression analysis (QRA) and multiple quadratic regression analysis (MQRA) were used to develop different equations to establish the inherent relationship between all investigated phenylpropanoid triggering enzymes and phenolic compounds in common bean sprouts. Also, partial correlation analysis was carried out to establish the relationship between all

investigated stress markers, phenylpropanoid triggering enzymes and accumulated phenolic compounds in common bean sprouts. From Table 4.2, we observed strong correlation coefficients between stress markers and activities of PAL and TAL. Since the presence of stress markers led to the expression of PAL and TAL, we further established the correlations between these factors (Table 4.3). Correlation analysis suggested that, irrespective of elicitor type PAL activity had high significant positive correlations with H_2O_2, CAT and POD, with correlation coefficients of 0.646, 0.795, and 0.677, respectively. Similarly, TAL activity significantly correlated with CAT, with a correlation coefficient of 0.539.

Table 4.2: Correlation between phenylpropanoid triggering enzymes and stress markers

Phenolic triggering enzyme	Stress marker		
	H_2O_2	CAT	POD
PAL	0.65	0.80	0.68
TAL	0.21	0.54	0.31

PAL – Phenylalanine ammonia-lyase; TAL - Tyrosine ammonia-lyase; H_2O_2 - Hydrogen peroxide; CAT-Catalase; POD – Peroxidase

Table 4.3: Correlation between phenylpropanoid triggering enzymes and phenolic compounds

Variable	Phenolic triggering enzyme	
	PAL	TAL
TPA	0.85	0.51
TFC	0.79	0.58

PAL – Phenylalanine ammonia-lyase; TAL - Tyrosine ammonia-lyase; TPA- Total phenolic acids; TFC-Total flavonoids content

However, activity of TAL was insignificantly correlated with H_2O_2, presenting a correlation coefficient of 0.156. These results indicate that, when sprouts are elicited, stress elements are generated leading to the stimulation of antioxidant (CAT and POD) enzymes and phenylpropanoid triggering enzymes (mainly PAL) for sprout defense and subsequent biosynthesis of defensive phenolic compounds, respectively.

Regression equations predicted from QRA and MQRA were used to establish the relationship between phenylpropanoid triggering enzymes and accumulated phenolic compounds investigated in this study (Table 4.5), where PAL, TAL, TPA and TFC represent phenylalanine ammonia-lyase, tyrosine ammonia-lyase, total phenolic acids and total flavonoids, respectively. From the predicted QRA equation (Eq. 4.5 and 4.6), irrespective of treatments, biosynthesis of TPA can reliably be predicted with data on activities of PAL and TAL. The R^2 of the regression of PAL and TAL activities were 0.72 and 0.55, respectively. Although, R^2 reported for both PAL and TAL activities were significant, it is important to point out that biosynthesis of TPA was rather highly associated with PAL activity, compared to TAL. Furthermore, to investigate the simultaneous relationship between activities of PAL and TAL during sprouting, we obtained a higher R^2 of 0.78 from the MQRA equation (Table 4.4 and Eq. 4.9) suggesting that, although PAL may be the dominating enzyme for triggering TPA biosynthesis in common bean sprouts, the presence of TAL also contributes a significant synergistic relationship. For instance, although this study reported a decrease in PAL activity for 100 mM NaCl elicited sprouts at 96 h, there was significant increase in their TAL activity at the same sprouting time, which may have accounted for its 65.03% significant increase of TPA at the same sprouting time.

Table 4.4: Predictive regression equations for phenylpropanoid triggering enzymes

Variable	Predictive equation	R^2	Eq. No.
QRA			
PAL	$TPA = 46.94 – 111.71PAL + 74.21PAL^2$	0.72	4.5
TAL	$TPA = -92.96 + 41.67TAL + 287.24TAL^2$	0.55	4.6
PAL	$TFC = 37.03 – 63.33PAL + 62.06PAL^2$	0.64	4.7
TAL	$TFC = 147.50 -659.58TAL + 861.71TAL^2$	0.61	4.8
MQRA			
PAL; TAL	$TPA = -146.29 + 209.53TAL + 28.55PAL^2$	0.79	4.9
PAL; TAL	$TFC = -163.18 + 289.95TAL + 27.70PAL^2$	0.75	4.10

PAL – Phenylalanine ammonia-lyase; TAL - Tyrosine ammonia-lyase

Similarly, predicted QRA equations established from this study showed a significant relationship between PAL activity and TFC accumulations, compared to TAL activity and TFC levels, with PAL; TFC and TAL; TFC reporting R^2 values of 0.64 and 0.61, respectively. However, from Eq. 4.10, MQRA seemed to predict a strong significant synergistic relationship between PAL and TAL in influencing biosynthesis of TFC by presenting an R^2 value of 0.75. These predictive relationships suggest that although PAL and TAL may perform differently under varied elicitation conditions during sprouting, maximum biosynthesis of phenolic compounds could be achieved in their synergy than under isolated conditions. Moreover, correlation coefficients between PAL and TPA; PAL and TFC; TAL and TPA; TAL and TFC were significantly 0.849, 0.789, 0.513 and 0.582, respectively (Table 4.3), further supporting predictive equations obtained from this study. Overall, MQRA predictive equations may be considered to better predict accumulations of phenolic compounds, with available data on PAL and TAL activities, during common bean sprout elicitations.

4.5 Conclusion

From this study, elicited common bean sprouts of 5 mM glutamic acid at 96 hr of sprouting presented the greatest accumulation of phenolic compounds and antioxidant capacities. Glutamic acid elicitations significantly elevated stress markers, which correlated with subsequent maximum expression of defense phenylpropanoid triggering enzymes (PAL and TAL). Most importantly, predicted MQRA equations led to the conclusion that, peak phenolic compounds can be accumulated in common bean sprouts under synergistic activities of PAL and TAL than in their isolated activities. Thus, elicitation can be used as an alternative natural mechanism to improve biosynthesis of phenolic compounds during sprouting of common beans.

CONNECTING STATEMENT TO CHAPTER V

Results from Chapter IV showed enhanced accumulation of phenolic compounds in sprouts elicited with glutamic acid, compared to NaCl and control treatments. In chapter V, the effect of ultrasonication in creating wounds, inducing formation of free radicals and stimulation of the phenylpropanoid pathway were investigated at different sprouting stages. Also, the effect of ultrasonication on accumulation of phenolic compounds and antioxidant capacities were determined. This chapter has been published in the Journal of Ultrasonics Sonochemistry as follows:

Ampofo, J. O. and Ngadi, M. (2020). Ultrasonic assisted phenolic elicitation and antioxidant potential of common (*Phaseolus vulgaris*) sprouts. *Ultrasonics Sonochemistry, 64* (2020), 104974.

CHAPTER V

Ultrasonic assisted phenolic elicitation and antioxidant potential of common bean (*Phaseolus vulgaris*) sprouts

5.1 Abstract:

Limited literature is available concerning the biosynthesis of phenolic compounds and antioxidative potential of common bean sprouts induced by ultrasound elicitation. In this study, common bean seeds were treated with ultrasound at different power (0, 180 and 360 W) and time (0, 30, 45 and 60 min) levels, before they were subjected to sprouting (24, 48, 72 and 96 h). Stress markers (structural defragmentation, H_2O_2, catalase and guaiacol peroxidase), activities of defense phenylpropanoid triggering enzymes (phenylalanine ammonia-lyase and tyrosine ammonia-lyase), phenolic compounds (total phenolic acids, total flavonoids and anthocyanins) and antioxidant capacities (DPPH, ABTS and Fe^{2+} scavenging) were monitored. Results showed that, ultrasound elicitation (especially 360 W, 60 min) significantly increased accumulation of stress markers at 96 h of sprouting, leading to elevated activities of defense phenylpropanoid triggering enzymes, phenolic compounds and antioxidant capacities at significant levels compared to the control. Ultrasound treatment at 360 W and 60 min reduced sprouting time by 60 h, compared to the control. Results from principal component analysis clearly differentiated latter stages of sprouting and high ultrasound levels from other investigated conditions as the distinct treatment conditions to maximize the production of phenolic compounds during sprouting of common beans. Also, SEM images showed increased cotyledon and seed coat porosity with increasing ultrasonic intensity. Overall, results from this study indicated that

elicitation with ultrasound can be a green and novel approach for producing phenolic-enriched common bean sprouts.

5.2 Introduction

Pulses such as common beans (*Phaseolus vulgaris*) have contributed to human nourishment since ancient time. In recent times, common beans are recognized as part of a healthy diet due to their high composition of protein, resistant starch and dietary fiber. Although common beans are nutrient-dense, some popular cultivars are limited in their concentration of phenolic compounds. These compounds, which are benzene ringed secondary metabolites with hydroxyl substituents, are produced in plants as defense compounds against environmental stress. Notable examples of phenolic compounds include phenolic acids, flavonoids, anthocyanins, tannins and coumarins (Liu et al., 2016). Consumption of phenolic-rich foods has been linked to positive health outcomes such as antioxidative, anti-cancer, anti-diabetic, anti-inflammatory, anti-microbial activities, etc., (Curran, 2012; Carvalho-Costa et al., 2015). Considering the wide consumption of common beans, enhancing their composition of phenolic compounds would have significant wide health benefits.

Depending on variety, environmental conditions, developmental stage, storage and processing conditions, different common beans have varied levels of phenolic compounds. For instance, Sutivisedsak et al. (2010) reported that navy, pinto, small and black beans have total phenolic acid levels of 8.34, 52.90, 10.30 and 63.80 mg GAE/g, respectively. In another study, black jamapa and pinto beans were reported to contain total flavonoid levels of 0.69 and 1.27 mg CE/g, respectively (Aparicio-Fernandez et al., 2005; Akillioglu and Karakaya, 2010). Due to these variations, there have been different studies aimed at improving phenolic compounds in common beans (Limon et al., 2014; Mendoza-Sanchez et al., 2016), especially in varieties with

lower levels. One simple approach is the induction of sprouting in seeds. Sprouting is a biochemical process that leads to two major outcomes namely the breakdown of stored complex molecules into simple forms and *novo* synthesis of non-existing compounds (Quinhone and Ida, 2015).

Although sprouting has been reported to increase concentrations of phenolic compounds in seeds, it is a slow process. Thus, there is a need to improve the rate of the sprouting process by application of appropriate technology based on the principle of the biosynthesis of phenolic compounds. Elicitation is the science of introducing plant systems to elicitors that can induce stress along the physiological and metabolic process chains, leading to the triggering of defense pathways and final accumulation of defense phenolic compounds at maximum levels (Liu et al., 2019).

Different forms of elicitors including biotic (e.g., microbial, organic acids, hormones etc.,), and abiotic (e.g., light, temperature, salts, hypoxia, etc.,) types are documented in literature to have elevated levels of phenolic compounds in treated sprouts, compared to their untreated forms (Ramakrishna et al., 2017). An elicitor should be sustainable, cheap, generally considered as safe (GRAS) and have consumer acceptance. A simple potential technology meeting these criteria is ultrasound (Yang et al., 2015). Application of ultrasound elicits cavitation, gas formation or vapor bubbles in plant-based foods, leading to oxidative stress and final triggering of defense phenolic compounds (Ding et al., 2018). For instance, Yu et al. (2016) reported increased resveratrol contents in different cultivars of ultrasonic-treated peanut sprouts, compared to their control. Similarly, Yang et al. (2015) reported on ultrasound treated soybean sprouts with 28.10, 43.40 and 49.21 % significant increases of daidzein, GABA and genistein, compared to the control. To the best of our knowledge, there is no literature that has investigated

how elicitation with ultrasound induces stress and stimulates defense phenylpropanoid triggering enzymes (Yu et al., 2016; Bormashenko et al., 2012; Alexia et al., 2001). The objective of this study was to investigate ultrasound elicited biosynthesis of phenolic compounds in common bean sprouts; elucidate induction of stress markers as correlated with microstructural disruptions; activities of defense phenylpropanoid triggering enzymes and subsequent accumulations of phenolic compounds; antioxidative potential of phenolic compound extracts obtained from ultrasound treated sprouts. Results of this study will be helpful in modulating processing systems for the production of phenolic-rich common bean sprouts.

5.3 Materials and methods

5.3.1 Common bean seeds

Common bean cultivar 'Kabulengeti' was provided by the Council for Scientific and Industrial Research Center of Zambia. This cultivar is by far the most widely available and consumed common bean in Zambia and most of East Africa. The cultivar has high yield stability; high tolerance to diseases and pests; and it has appreciable protein and lipid contents of 24.38 and 1.38 %, respectively. Despite its nutrient and high yield properties, kabulengeti bean has low levels of phenolic acids (3.76 mg CE/100 g) and flavonoids (2.24 mg RE/100 g), thus, it was selected for this study as a representative of common bean cultivars with limiting levels of phenolic compounds.

5.3.2 Ultrasound treatment

All processing surfaces were sanitized by washing with 1% sodium hypochlorite prior to use. Approximately 100 g of common bean seeds were disinfected with 500 mL of 1% sodium

hypochlorite for 20 min and washed with distilled water until neutral pH was attained. The disinfected bean seeds were transferred into a specially designed seed holder and treated with ultrasound at power levels of 360 and 180 W and time levels of 30, 45 and 60 min at 25ºC, using an ultrasonic cleaner bath (Ultrasonic model: TRTV1847, HF-Pk-power: 360 W, 40 kHz, capacity: 15L, 11.8 x 13 x 6 inches, weight: 9.08 Kg, Beijing Ultrasonic, Beijing, China). Ice cubes were added intermittently during ultrasound treatments to prevent temperature rise and imbalance. After ultrasonication, each bean sample was sprouted as described below in section 5.3.3. Control seeds were disinfected and soaked in distilled water (1:3 w/v) for 60 min at 25ºC without ultrasonication before they were subjected to sprouting.

5.3.3 Common bean sprout preparation

Ultrasonic treated and control bean seeds were sprouted in darkness at 25ºC for 24, 48, 72, and 96 h in an incubator (New Brunswick Scientific Co. Inc. Edison, N.J., USA) on petri dishes lined with absorbent paper. During the sprouting period, the seeds were sprayed with 25 mL of distilled water at intervals of 6 h. After each sprouting time, sprouts were collected, studied for their morphological characteristics, washed and frozen for lyophilization. Lyophilized sprouts were milled using a Cyclone Sample Mill (UDY Corp., Fort Collins, CO, USA) fitted with a 1 mm screen, and used for analysis. Sprouting was performed in duplicates for each treatment.

5.3.4 Evaluation morphological characteristics

5.3.4.1 Physical parameters

To evaluate the influence of ultrasound on the physical properties of common bean sprouts, radicle length and hypocotyl diameter of the sprouts were measured. Radicle length (cm)

was determined at each 12 h by measuring from the tip of the radicle to the base of the hypocotyl with a measuring ruler. Hypocotyl diameter (mm) was determined by measuring the width of hypocotyl with a Vernier caliper.

5.3.4.2 Sprouting potential

Sprouting percentage, sprouting index and sprout vigor index were evaluated after every 12 h, and related to sprouting potential (Bormashenko et al., 2012; Ling et al., 2014):

Sprouting percentage (%): $S_p = N_t/N_{TS}$ Eq. (5.1)

Sprouting index: $S_i = \sum N_{Dt}/t$ Eq. (5.2)

Sprout vigor index: $S_{vi} = S_i \times L$ Eq. (5.3)

where S_p, S_i *and* S_{vi} are sprouting percentage, sprouting index and sprout vigor index, respectively. N_t is the number of total sprouts showing emerged radicle; N_{TS} is the total number of seeds on the petri dish; N_{Dt} is the number of sprouted seeds at time t; t is the sprouting time *and* L is the total length of the sprout.

5.3.5 Determination of stress markers

5.3.5.1 Hydrogen peroxide (H_2O_2) analysis

Accumulation of H_2O_2 was evaluated as described by Alexieva et al. (2001). Approximately 0.1 g of sample flour was homogenized with 1 mL of mixed solution (0.25 mL of 0.1% TCA, 0.5 mL of 1M KI and 0.25 mL of 10 mM potassium phosphate buffer, pH 7.0) at 4°C for 10 min. Afterwards, the obtained mixture was centrifuged (10000 g, 15 min, 4°C), the supernatant kept in the dark for 30 min and absorbance measured at 390 nm with a microplate reader. A control was prepared by replacing sample with 0.1% TCA in the reaction mixture.

5.3.5.2 Stress indicative enzymes

5.3.5.2.1 Extract preparations and protein content determination

5.3.5.2.2 Total protein assay

Protein contents of enzyme extracts were measured as described by Bradford (1976). Bradford reagent was prepared by diluting the dye concentrate in a 1:4 ratio with distilled water. To 20 µL of enzyme extract, 1 mL of the prepared Bradford reagent was added, incubated at room temperature for 5 min, vortexed for 5 sec, and absorbance measured at 595 nm using a microplate reader. For control, enzyme extract was replaced with extraction buffer and subjected to the same conditions described above. Protein assay was performed on the same day of extraction.

5.3.5.2.3 Enzyme extraction

Extracts of catalase (CAT) and guaiacol peroxidase (GPX) were performed according to the method of Swieca, Seczyk and Gawlik-Dziki (2014). Approximately, 200 mg of sample flour was homogenized at 4°C for 20 min with 4 mL of 100 mM sodium phosphate buffer (pH 6.4) containing 0.2 g of polyvinylpyrrolidone. Afterwards, the homogenized mixture was centrifuged (12000 g, 4°C, 30 min), and the supernatant was collected as enzyme extract. Enzymatic assays were performed on the same day of extraction.

5.3.5.2.4 Enzymatic assays

For catalase (CAT) activity, the reaction mixture comprised of 0.05 mL enzyme extract and 0.95 mL of H_2O_2 (10 mM, in 100 mM sodium phosphate buffer, pH 7). Decomposition of H_2O_2 was monitored by a decrease in absorbance at 240 nm after 1 min at 30°C. One unit was

defined as the amount of catalase capable of decomposing 1.0 µmol H_2O_2 per min under the conditions of the assay. Results were expressed as U/mg of protein (Swieca, Seczyk and Gawlik-Dziki, 2014).

Assay of guaiacol peroxidase (GPX) was performed by the method of Burguieres et al. (2007) with slight modifications. The reaction mixture was made up of 0.1 mL enzyme extract, 2 mL guaiacol (8 mM, in 100 mM sodium phosphate buffer, pH 6.4) and 1 mL H_2O_2 (24 mM), incubated for 1 min at 30°C, and the change in absorbance was measured at 460 nm with a microplate reader. GPX activity was expressed as U/mg of protein, where 1U = 0.001 Δ460 nm/min under the conditions of the assay.

5.3.6 Evaluation of defense phenylpropanoid triggering enzymes

5.3.6.1 Enzyme extraction

Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) extractions were performed at 4°C. Briefly, 200 mg of sample flour was homogenized with 2 mL extracting buffer (0.2 M boric acid buffer containing, 1 mM EDTA and 50 mM β-mercaptoethanol, pH 8.8) for 20 min, centrifuged (12000 g, 4°C, 30 min) and the supernatants collected as enzyme extracts (Swieca, Seczyk and Gawlik-Dziki, 2014). Enzymatic assays were performed on the same day of extraction.

5.3.6.2 Enzymatic assays

Analysis of PAL was evaluated by incubating the reaction mixture (300 µL enzyme extract, 1.2 mL of 0.02 M L-phenylalanine and 2 mL PAL extracting buffer) at 30°C for 60 min. After incubation, reaction was stopped by adding 0.5 mL of 10% TCA, centrifuged (15000 g, 10

min, 4°C) and absorbance of the supernatant measured at 290 nm with a microplate reader. One unit was defined as the amount of PAL that produced 1.0 µmol trans-cinnamic acid per min under conditions of the assay. Results were expressed as U/mg of protein (Assis et al., 2001).

For tyrosine ammonia-lyase (TAL) assay, the reaction mixture (100 µL enzyme extract, 0.9 mL of 0.02 M L-tyrosine) was incubated at 30°C for 60 min. Afterwards, reaction was stopped by adding 0.5 mL of 10% TCA, centrifuged (15000 g, 10 min, 4°C) and absorbance of the supernatant read at 310 nm with a microplate reader. One unit was defined as the amount of TAL that produced 1.0 µmol *p*-coumaric acid per min under the assay conditions. Results were calculated as U/mg of protein (Assis et al., 2001).

5.3.7 Assessment of phenolic compounds

5.3.7.1 Extraction of phenolic compounds

Phenolic compounds were extracted as described by Marathe et al. (2011). About 1 g of sample flour was extracted with 15 mL of 80% aqueous methanol (V/V), centrifuged (5000 rpm, 10 min), and the supernatant filtered. Afterwards, methanol in the supernatant was evaporated using a rotary evaporator and obtained phenolic compound extract was freeze-dried for subsequent analysis.

5.3.7.2 Determination of total phenolic acids and anthocyanins

Phenolic compound freeze-dried extracts were dissolved in 80% methanol and filtered through 0.45 µm cellulose acetate filter (Millipore) prior to analysis. Exactly 20 µL of the dissolved sample phenolic compound extract was mixed with 240 µL of 2% HCL in 75% methanol, vortexed for 1 min and absorbances read with a microplate reader at 280 and 520 nm

for total phenolic acids and anthocyanins, respectively. Standard curves were prepared under similar conditions with (+)-catechin (0-200 μgmL^{-1}) and cyanidin-3-glucoside (0-20 μgmL^{-1}) for quantitative measurements of total phenolic acids and anthocyanins, respectively. Total phenolic acids and anthocyanins were calculated as catechin and cyanidin-3-glucoside equivalents, in mg/100 g of sample flour, respectively (Mazza et al., 1999).

5.3.7.3 Determination of total flavonoids

Total flavonoids were measured according to the method of Hairi, Sallé and Andary (1991). Phenolic freeze-dried extracts were dissolved in 80% methanol and filtered through a 0.45 µm cellulose acetate filter (Millipore) prior to analysis. Exactly 50 µL of dissolved sample phenolic compound extract was mixed with 200 µL of 10 gL^{-1} 2-aminoethyldiphenylborate, vortexed for 1 min, and absorbance measured with a microplate reader at 404 nm. A standard curve for quantitative measurement was prepared under the same conditions using rutin (0-50 μgmL^{-1}). Total flavonoid content was calculated as rutin equivalents in mg/100 g of sample flour.

5.3.8 Antioxidant activities

5.3.8.1 Estimation of DPPH free radical scavenging capacity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging capacity was measured according to the method of Akond et al. (2011). Briefly, 83 µL of phenolic compound extract was mixed with 167 µL of 0.1 mM DPPH solution, vortexed for 1 min, kept in the dark for 30 min, and absorbance measured with a microplate reader at 517 nm. A control reaction mixture was prepared by replacing phenolic compound extract with 80% methanol under the same

conditions. Ability of sample phenolic compound extract to scavenge DPPH free radical was determined using Eq. 5.4 below:

$$\% \text{ Scavenging activity} = \frac{A_{control} - A_{test}}{A_{control}} \times 100 \qquad \text{Eq. (5.4)}$$

5.3.8.2 Estimation of ABTS scavenging capacity

ABTS scavenging capacity was determined as described by Re et al. (1999). ABTS free radical solution was prepared by adding 5 mL of 14 mM ABTS solution to 5 mL of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution and stored in the dark for 16 h at 25°C. Prior to using this solution, it was diluted with distilled water to get an absorbance of 0.900 ± 0.020 at 734 nm. Final reaction mixture comprised of 200 µL ABTS free radical solution and 50 µL sample phenolic compound extract, vortexed for 1 min, kept in the dark for 6 min and absorbance measured with a microplate reader at 734 nm. A control reaction mixture was prepared by replacing phenolic compound extract with 80% methanol under the same conditions. The ability of a sample phenolic compound extract to scavenge ABTS free radical was determined using Eq. 5.4.

5.3.8.3 Estimation of ferrous (Fe^2) chelating capacity

Chelation of ferrous ions by phenolic compound extracts was estimated by the method of Dinis, Madeira, and Almeida (1994). Briefly, 50 µL sample phenolic extract was added to 0.002 M Fe (II) chloride (25 µL) and 0.005 M ferrozine (200 µL). After incubating in darkness at 25°C for 10 min, absorbance of the mixture was measured at 562 nm with a microplate reader. Ferrous chelating capacity of a sample phenolic compound extract was determined using Eq. 5.4.

5.3.9 Scanning electron microscopy analysis

To investigate the effect of ultrasonication on the microstructure of inner and outer surfaces of cotyledon and seed coat, scanning electron microscope (Hitachi TM-3000, Hitachi, Japan) was used. Briefly, microstructural images were obtained by fixing dried samples on an adhesive specimen holder and inserted into scanning electron microscope at 5 kV acceleration voltage.

5.4 Results and discussion

5.4.1 Impact of ultrasound on morphological characteristics of common bean sprouts

5.4.1.1 Radicle elongation and hypocotyl diameter

Ultrasound treatments at both 360 and 180 W showed radicle emergence at 24 h of sprouting with significant radicle elongation at increasing sprouting times, whereas control treatments seemed to delay radicle emergence up to 48 h of sprouting (Figure 5.1 a and b). Overall, the highest radicle length of 7.65 cm was elicited with the most intense ultrasound treatment (360 W; 60 min) which was significantly ($P < 0.05$) higher by 16.88 and 72.37 % when compared to 180 W (60 min) and control treatments, respectively, at the same sprouting time of 96 h. Thus, ultrasound treatment enhanced radicle growth with increasing treatment intensity. Gagliano, Stefano, and Daniel (2012) reported significant increments of radicle elongations in sprouted *Zea mays* when exposed to sound waves, compared to its control. According to Hassanien et al. (2014) exposure of plant cells to ultrasonic waves can stimulate cell divisions and activities of endogenous hormones. Auxins is the key hormone responsible for inducing meristematic activities in radicle and root tips for elongation (Simon and Petrášek, 2011). The

result obtained in this study may be attributed to an increased expression of auxins in the radicle tip at early sprouting stages, in response to ultrasound treatments.

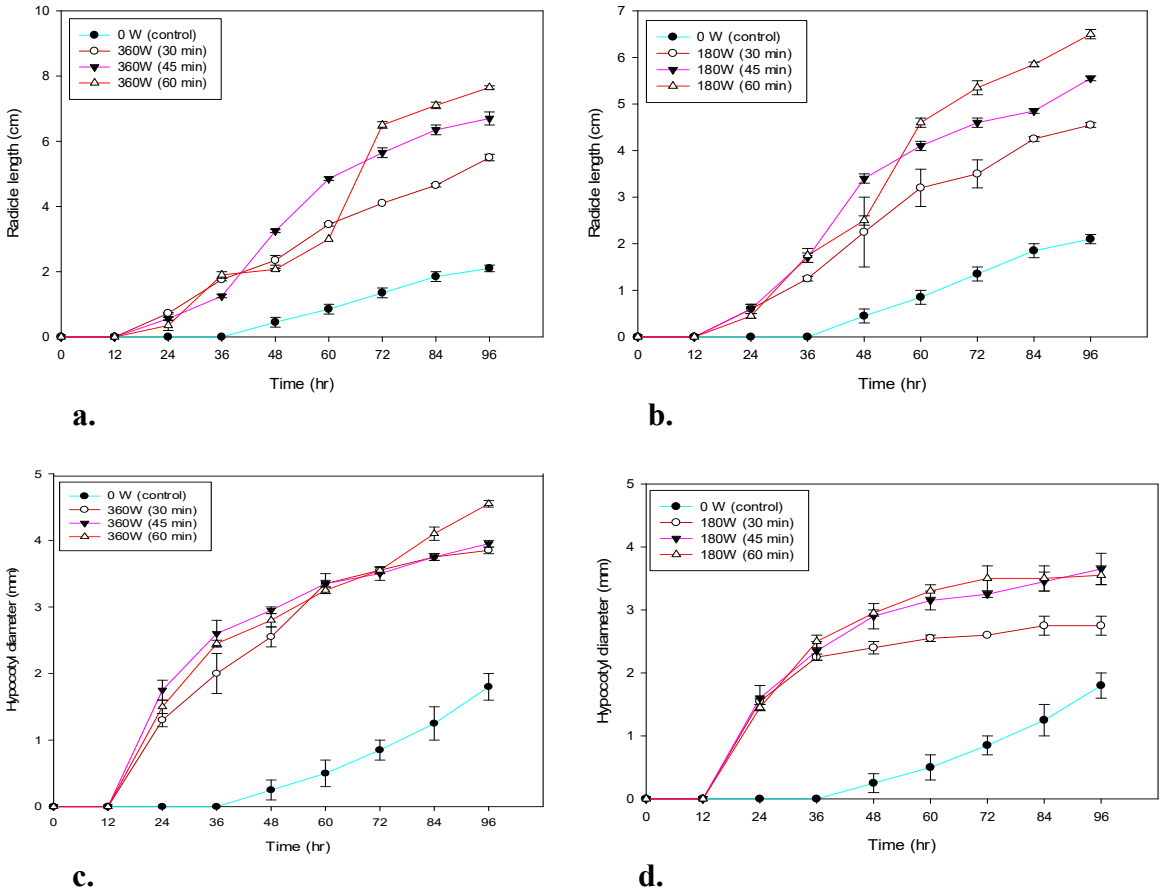


Figure 5.1(a-d): Effect of 360 W (a, c) and 180 W (b, d) ultrasound treatments on radicle and hypocotyl growths of common bean sprouts. Results are presented as mean ± SD of three independent experiments

Similar to radicle growth, hypocotyl of ultrasonic treated sprouts emerged from the seed coat at 24 h of sprouting, compared to the control which showed a delay in hypocotyl emergence until 48 h of sprouting (Fig. 5.1 c and d). Again, treatment with higher intensity (360 W; 60 min) of ultrasound elicited the maximum hypocotyl growth of 4.55 mm at 96 h of sprouting which was 60.44% significantly ($P < 0.05$) higher than the control, at the same sprouting time.

5.4.1.2 Effect of ultrasound treatment on sprouting percentage, sprouting index and vigor index

Seeds were defined as sprouts with the emergence of the radicle from the seed coat. The results show that ultrasound significantly enhanced sprouting percentage (Eq. 5.1) of common beans, with intensification at high ultrasound power and time levels. For ultrasonic treatments, no sprouting was observed at the initial 12 h, whereas for control sprouting was delayed for much longer than 36 h. (Figure 5.2 a, b). However, the higher the ultrasound treatment, the higher the increase in sprouting percentages of common beans. This result is consistent with results on radicle growth since bean seeds were considered as sprouts on emergence of radicle from the seed coat. The maximum sprouting percentage of 100% was elicited at 36 h of sprouting with 360 W (60 min) ultrasound treatment and was comparatively 22 or 100 % higher than the results obtained with either the lower intensity of 180 W (60 min) and the control treatments, respectively, at the same sprouting time. Further, treatment at the higher ultrasound intensity of 360 W (60 min) saved sprouting time by 24 or 60 h compared to treatment at 180 W (60 min) or the control, respectively, at the same sprouting time.

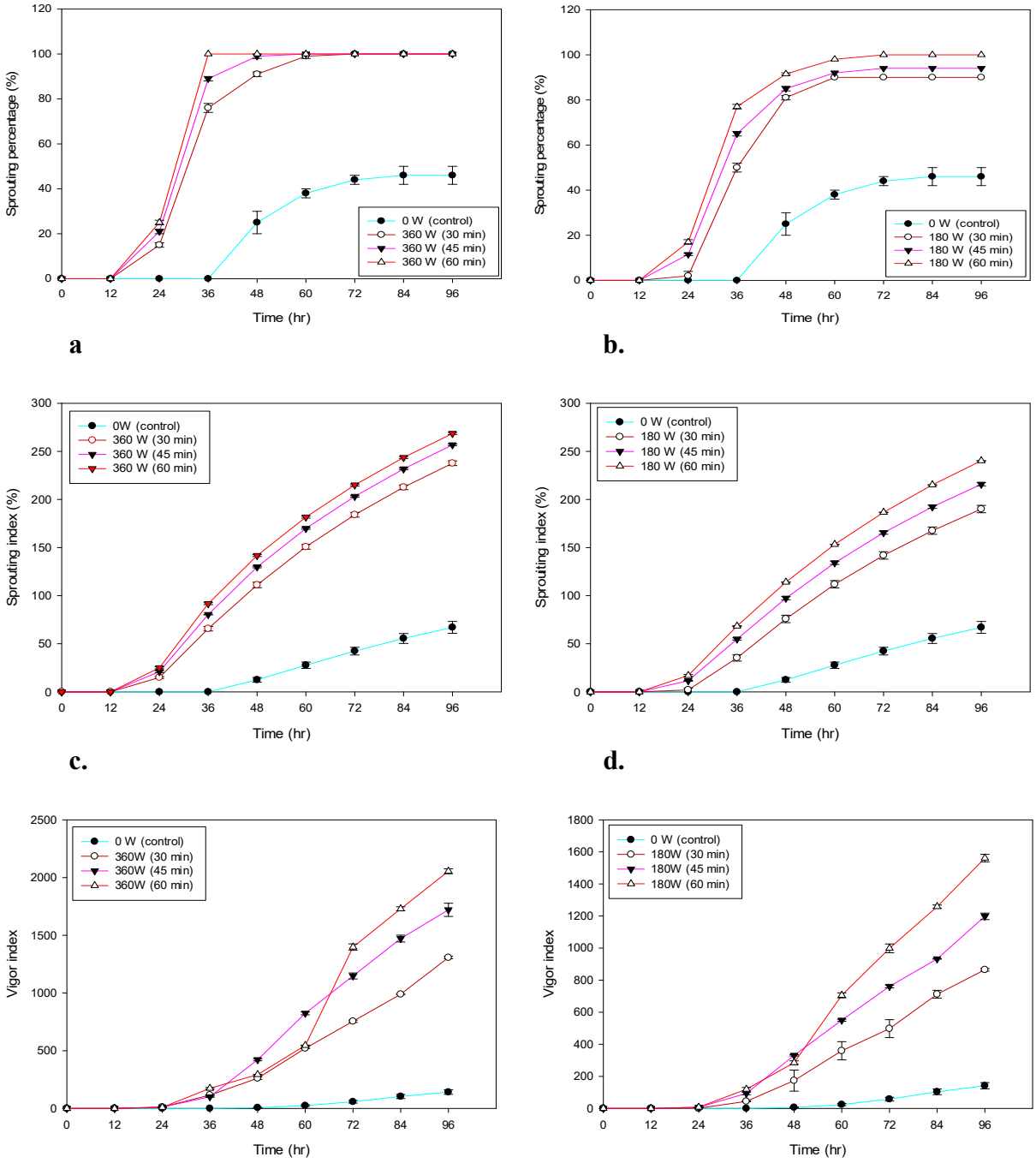


Figure 5.2(a-f): Effect of 360 W (a, c, e) and 180 W (b, d, f) ultrasound treatments on sprouting percentage, sprouting index, and vigor index of common bean sprouts. Results are presented as mean ± SD of three independent experiments

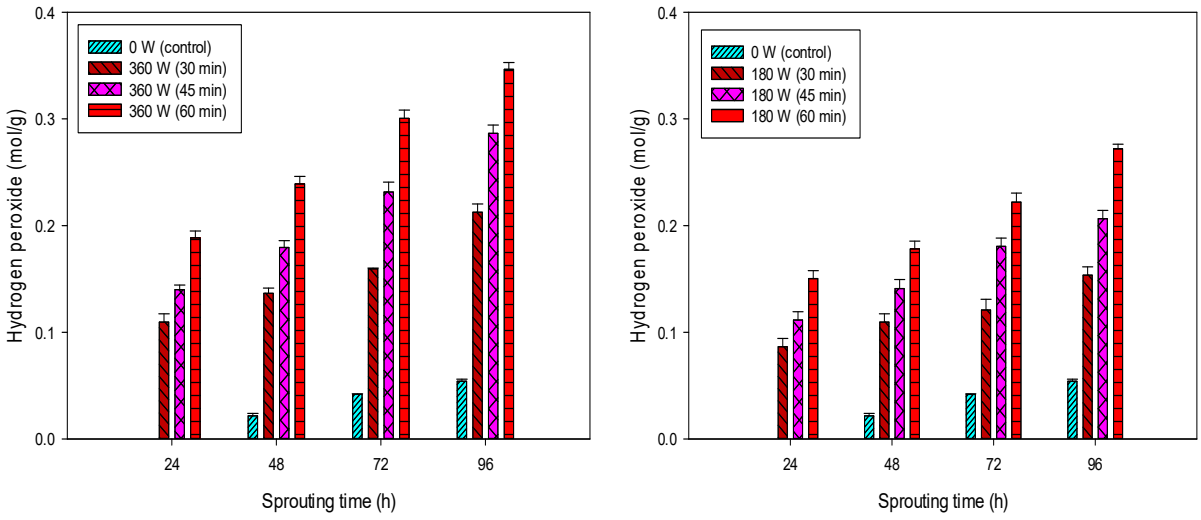
Ultrasound waves may have transferred energy to cells of growing radicle to overcome the seed coat barrier for early emergence. Wang et al. (2003) observed improved sprouting rate in ultrasound treated paddy rice and attributed their observation to the ability of ultrasound waves to transfer energy to cytoplasmic cells for efficient cytoplasmic streaming during the sprouting process. Similarly, Yu et al. (2016) reported 18.07% significant increases in sprouting percentage with ultrasound treated peanut sprouts compared to their control. According to Chen et al. (2013) ultrasonic vibration can increase biochemical activities by deforming cell wall structure and increasing permeability of membranes inside and outside seed cells. Furthermore, improved sprouting percentage by ultrasound may be due to the acoustic vibration weakening of the seed coat, causing changes in seed microstructure, leading to enhancements in seed water uptake, and release of hydrolytic enzymes otherwise embedded in intact cells for metabolic processes needed for efficient sprouting.

Apart from increasing sprouting percentage, ultrasonication significantly ($P < 0.05$) enhanced sprouting indexes and vigor of common beans, compared to the control as shown in Fig. 5.2 c, d and e, f. It should be highlighted that, sprouting index and radicle length were factors used to determine vigor index (Eq. 5.3), and in this study ultrasound significantly enhanced these factors compared to the control, thus explaining its maximum effects on sprout vigor index over that in the control. Conclusively, elicitation of common beans with ultrasound resulted in significant improvements of morphological characteristics compared to the control.

5.4.2 Accumulation of stress markers

5.4.2.1 Hydrogen peroxide (H_2O_2) levels

Acoustic cavitation created by ultrasound has been linked to the induction of stress in plant tissues through the production of oxidative free radicals such as H_2O_2. It was observed that generation of H_2O_2 in the bean sprouts was maximum at 0.35 mol/g after 96 h of sprouting with 360 W (60 min) (Fig. 5.3 a, b). This result was 1.30 and 7.70 times significantly ($P < 0.05$) higher than H_2O_2 levels obtained with 180 W (60 min) and control treatments, respectively, at the same sprouting time. This observation may be due to the possible breaching of seed cell wall and membrane integrities by ultrasound cavitation, which led to increased demand for metabolic activities responsible for repair of damaged structures, and subsequent elevation of H_2O_2 as metabolites of these processes. According to Ramachandra, Viswanatha, and Vivekanandan (2004), H_2O_2 are end-products of cellular metabolic activities, with increasing accumulations under stressful conditions.



a. **b.**

Figure 5.3(a-b): Effect of 360 W (a) and 180 W (b) ultrasound treatments on accumulation of hydrogen peroxide in common bean sprouts. Results are presented as mean ± SD of three independent experiments

Furthermore, elevated H_2O_2 with ultrasound treatments synchronized with enhanced observations of morphological characteristics with ultrasound. Thus, ultrasound treatment may have signaled accumulations of H_2O_2 at growth promoting levels, rather than at lethal degradative levels along the sprouting process. Çavuşoğlu and Kabar (2010) reported that H_2O_2 at low threshold levels are non-toxic, but rather promote sprouting by helping break seed dormancy, and signaling the release of hormones involved in the sprouting process.

5.4.2.2 Catalase (CAT) and guaiacol peroxidase (GPX) activities

CAT is an antioxidant enzyme that decompose H_2O_2 into water and oxygen (Agrawal, Joshi, and Subramani, 2011). To the best of our knowledge, no work has been reported on the activity of CAT with ultrasound elicitation along the sprouting process of common beans. Compared to control treatments, ultrasonication significantly elicited CAT activity at the beginning of sprouting, with this trend increasing as sprouting prolonged (Fig. 5.4 a-b). The highest CAT activity of 26.04 U/mg protein was elicited at the highest ultrasound intensity, 360 W (60 min), at 96 h of sprouting. This result was 24.81 and 89.17 % higher than CAT activities elicited with lower ultrasound level of 180 W (60 min) and the control, respectively.

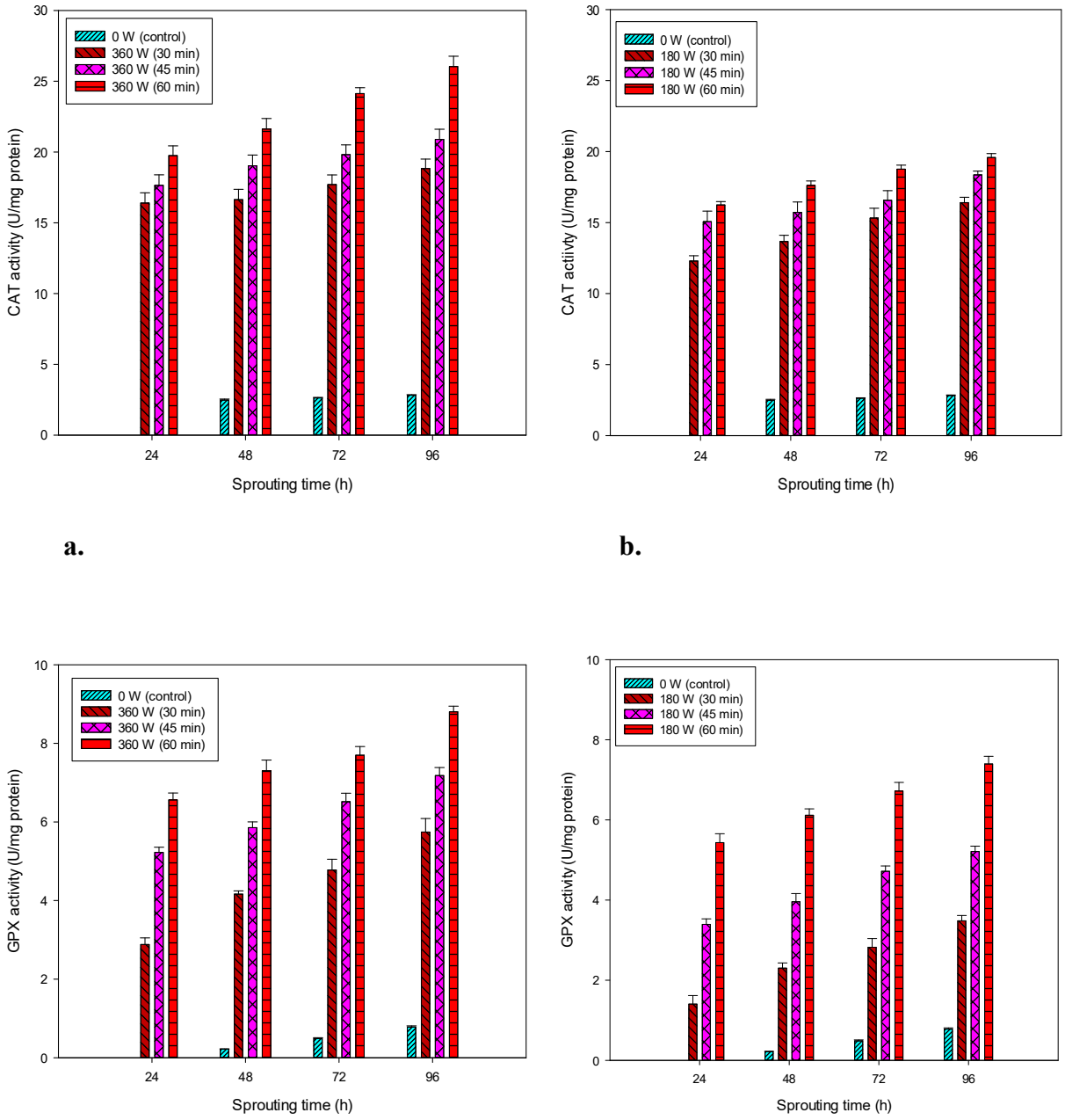


Figure 5.4(a-d): Effect of 360 W (a, c) and 180 W (b, d) ultrasound treatment on activities of catalase (CAT) and guaiacol peroxidase (GPX) in common bean sprouts. Results are presented as mean ± SD of three independent experiments

These results suggest that, oxidative stress increased significantly with ultrasonication and sprouting time, principally under ultrasonic conditions of high power and time, leading to increasing demand for CAT to decompose formed H_2O_2 and protect sprout cells from oxidative stress. Furthermore, this result points out that the majority of H_2O_2 that were elicited by ultrasound were decomposed by CAT, thus explaining the non-lethal levels of H_2O_2 against morphological characteristics of common bean sprouts reported in this study.

Guaiacol peroxidase (GPX) is the enzyme that catalyzes the transformation of monophenols into structural polyphenols (lignin) needed for cell wall development, structuring and repair (Erofeeva, 2015). From this study, ultrasound induced activity of GPX from the onset of sprouting and increased significantly ($P < 0.05$) as sprouting was prolonged (Fig. 5.4 c-d). For control samples, GPX activity became apparent from 48 h of sprouting with no significant increases with sprouting time. The greatest GPX activity of 8.80 U/mg protein was elicited with 360 W (60 min) ultrasound treatment at 96 h of sprouting and was 15.98 and 91.14 % significantly higher than the GPX activity obtained with the 180 W (60 min) and control treatments, respectively, at the same sprouting time. Elevated GPX activity with ultrasonic treatments signify elevated demand for sprout cell wall development and repair, possibly due to the disruptive cavitation effect of the applied ultrasonic waves before initiating sprouting.

5.4.3 Defense phenylpropanoid triggering enzymes

5.4.3.1 Activities of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL)

PAL is rated as the key enzyme responsible for triggering the phenylpropanoid pathway, the metabolic pathway associated with the biosynthesis of plant phenolic compounds (Pichyangkura and Chadchawan, 2015). Ultrasound treatments maximally elicited activities of

PAL throughout the sprouting process (Fig. 5.5 a, b). Control treatment only showed PAL activity at 48 h of sprouting with no significant increases afterwards. Similar to PAL, the greatest activity of TAL was also elicited with 360 W (60 min) ultrasound treatment at 96 h of sprouting (Fig. 5.5 c-d).

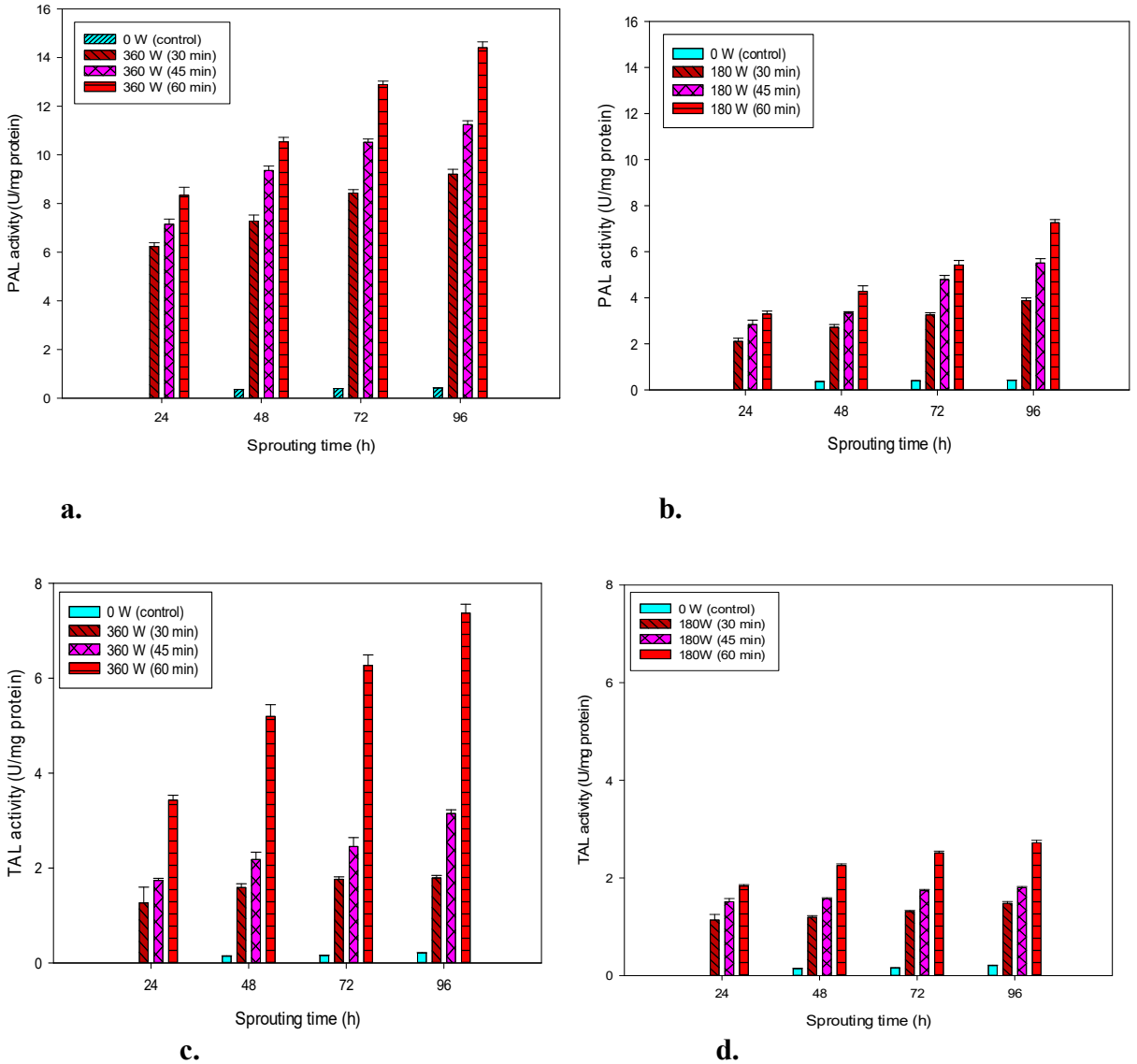


Figure 5.5(a-d): Effect of 360 W (a, c) and 180 W (b, d) ultrasound treatments on activities of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) in common bean sprouts. Results are presented as mean ± SD of three independent experiments

Enhancement of PAL and TAL activities with ultrasound agreed with the results on stress markers (H_2O_2, CAT and GPX). Thus, there is indication that elevated stress induced with ultrasonic elicitation of common beans led to significant activities of phenylpropanoid triggering enzymes from the onset of sprouting and increased as sprouting prolonged. According to Mendoza-Sanchez et al. (2016) disruption of cell tissues leads to the accumulation of H_2O_2 in cell wall, which further leads to the induction of phenylpropanoid triggering enzymes such as PAL, for the biosynthesis of phenolic compounds.

Also, enhanced activities of PAL and TAL with ultrasound treatments could be due to significant signaling of phenylalanine and tyrosine (substrates for PAL and TAL, respectively) during the sprouting process as reported by Yang et al. (2015), who observed significant increases for phenylalanine and tyrosine with ultrasound treated soybean sprouts, compared to their control counterparts. Furthermore, it should also be pointed out that activity of PAL was significantly elicited at higher levels, compared to TAL, confirming literature reports that PAL is the main enzyme actively involved in triggering the phenylpropanoid pathway towards the synthesis of phenolic compounds (Mendoza-Sánchez et al., 2016). This trend may be due to variations in phenylalanine (PAL substrate) and tyrosine (TAL substrate) availabilities along the sprouting process, as a result of ultrasound treatment. Phenylalanine which is the substrate for PAL is also the substrate for tyrosine biosynthesis (Chavez-Bejar et al., 2012), hence suggesting that high PAL activity will result in reduced phenylalanine availability for tyrosine biosynthesis and further reducing substrate availability for TAL activity.

5.4.4 Accumulation of phenolic compounds

5.4.4.1 Total phenolic acids, flavonoids and anthocyanins

Exposure of plant cells to elicitors induce various physiological processes that could lead to changes in composition and profile of phenolic compounds (Liu et al., 2019). Total phenolic acids, flavonoids and anthocyanins of sprouted samples increased significantly ($P < 0.05$) with ultrasound treatments, over their untreated forms (Table 5.1). The greatest contents of total phenolic acids (216.74 mg/100g) was accumulated with the highest ultrasound treatment, 360 W (60 min), at 96 h of sprouting. This value was higher by 2.6 and 11.65 folds when compared with the lower ultrasound intensity treatment of 180 W (60 min), and the control, respectively, at the same sprouting time.

Similarly, the highest total flavonoids content of 203.50 mg/100g was elicited with 360 W (60 min) at 96 h of sprouting; significantly higher by 1.7 and 6.6 folds when compared to 180 W (60 min) and control treatments, respectively, at the same sprouting time. Likewise the total anthocyanins value measured at 30.35 mg/100 g at 96 h of sprouting obtained with ultrasound elicitation of 360 W (60 min) was significantly higher by 1.46 and 11.54 folds compared to the values obtained with 180 W (60 min) and control treatments, respectively, at the same sprouting time. Similar to the current study, Yang et al. (2015) reported increased contents of daidzein and genistein for 300 W ultrasound elicited soybean sprouts by 28.13 and 49.21%, respectively, compared to their control counterparts.

Table 5.1: Effect of ultrasonic treatments on accumulation of phenolic compounds in common bean sprouts

Phenolic compound	Treatment	Sprouting time (h)			
		24 h	48 h	72 h	96 h
Total phenolic acids (mg/100 g)	Control	0.00±0.00^{a}	14.24±0.06^{a}	16.51±0.09^{a}	18.60±0.08^{a}
	US-360-30	21.05±0.44^{d}	28.26±0.73^{d}	32.31±0.12^{b}	176.45±1.58^{e}
	US-360-45	120.93±1.09^{e}	144.03±3.78^{e}	151.16±0.63^{f}	196.09±0.59^{f}
	US-360-60	127.05±3.41^{f}	156.10±5.49^{f}	159.21±1.34^{g}	216.74±1.26^{g}
	US-180-30	4.55±1.09^{c}	18.34±2.32^{b}	39.31±0.51^{c}	73.33±1.27^{b}
	US-180-45	1.97±1.09^{b}	22.39±0.73^{c}	42.81±0.19^{d}	78.71±0.15^{c}
	US-180-60	4.81±0.73^{c}	25.60±0.15^{c}	47.57±1.71^{e}	82.74±0.73^{d}
Total flavonoids (mg/100 g)	Control	0.00±0.00^{a}	24.64±1.13^{a}	26.84±0.85^{a}	30.84±0.85^{a}
	US-360-30	46.50±0.23^{e}	66.83±0.24^{e}	84.83±0.71^{d}	142.17±1.65^{e}
	US-360-45	51.83±1.18^{f}	71.77±0.79^{f}	91.45±0.63^{e}	191.17±0.24^{f}
	US-360-60	60.67±0.94^{g}	77.33±0.47^{g}	98.67±0.94^{f}	203.50±3.54^{g}
	US-180-30	31.50±0.24^{b}	41.83±1.18^{b}	49.17±0.71^{b}	89.83±0.71^{b}
	US-180-45	36.17±0.24^{c}	48.67±0.47^{c}	61.67±0.94^{c}	95.67±0.47^{c}
	US-180-60	42.00±0.94^{d}	57.33±0.47^{d}	86.00±2.36^{d}	119.17±0.24^{d}
Anthocyanins (mg/100 g)	Control	0.00±0.0^{a}	2.38±0.03^{a}	2.49±0.03^{a}	2.63±0.03^{a}
	US-360-30	9.87±0.07^{e}	10.71±0.10^{e}	13.34±0.21^{e}	17.44±0.49^{c}
	US-360-45	15.39±0.09^{f}	17.54±0.21^{f}	19.56±0.29^{f}	22.47±0.21^{e}
	US-360-60	16.96±0.03^{g}	20.35±0.41^{g}	24.84±0.04^{g}	30.35±0.13^{f}
	US-180-30	8.09±0.02^{b}	9.64±0.21^{b}	11.14±0.03^{b}	14.24±0.02^{b}
	US-180-45	10.96±0.04^{c}	12.44±0.01^{c}	14.72±0.17^{c}	17.98±0.02^{c}
	US-180-60	12.69±0.14^{d}	15.69±0.08^{d}	18.08±0.07^{d}	20.78±0.09^{d}

Data are presented as mean ± SD of three independent experiments. Different superscript letters within a column indicate significant differences ($p < 0.05$). US; ultrasound

As it has already been established in this study, elicitation of common beans with ultrasound increased the accumulation of stress markers from the onset of sprouting until the process was arrested, signifying a demand for sprout protection along the sprouting process. Thus, resulting in the elevated stimulation of defense phenylpropanoid triggering enzymes (PAL and TAL), and final biosynthesis of phenolic compounds at maximum levels compared to the control.

To our knowledge, this is the first time these connections are made to explain the accumulation of phenolic compounds in common bean sprouts. Results of this study suggest that ultrasound could be a simple sustainable approach to elicit stress at functional non-lethal levels towards the production of natural phenolic-dense common bean sprouts.

5.4.5 Antioxidant capacities

The ability of a compound to protect a biological system from the negative effect of oxidative stress is defined as its antioxidant capacity. Antioxidant capacities of phenolic compound extracts obtained from ultrasonic treated common bean sprouts were significantly ($P < 0.05$) higher compared to the control as presented in Table 5.2, with this trend increasing significantly over sprouting time.

Results of this study indicate that, the greatest capacity to inhibit reactivity of DPPH free radicals (97.81%) was observed with 360 W (60 min) ultrasound treatment at 96 h of sprouting, and was significantly higher by 5 and 13.84 %, compared to the 180 W (60 min) ultrasound and control treatments, respectively, at the same sprouting time. Similarly, the greatest capacity to scavenge ABTS free radicals (98.34%) was observed with 360 W (60 min) ultrasound treatment at 96 h of sprouting, with this result being 8.62 and 25.57 %, significantly higher than 180 W (60 min) ultrasound and control treatments, respectively, at the same sprouting time. Along with DPPH and ABTS free radicals scavenging, the maximum capacity to chelate ferrous ions for sprouts treated with 360 W (60 min) ultrasound at 96 h of sprouting was 7.39 and 30.79 % significantly higher than the corresponding capacities for 180 W (60 min) ultrasound and control treatments, respectively, at the same sprouting time. DPPH and ABTS free radicals are principally scavenged by non-polar (e.g., flavonoids and saponins etc.,) and polar (e.g., phenolic

acids) antioxidant phenolic compounds, respectively, by donating hydrogen and electron atoms to quench and stabilize these free radicals (Mendoza-Sánchez et al., 2016).

Table 5.2: Antioxidant potential of phenolic compounds extracts elicited from ultrasonic treated common bean sprouts

Antioxidant method	Treatment	Sprouting time (h)			
		24 h	48 h	72 h	96 h
DPPH (%)	Control	0.00±0.00^{a}	82.16±0.12^{a}	83.16±0.02^{a}	84.27±0.12^{a}
	US-360-30	89.46±0.19^{d}	90.25±0.09^{d}	91.27±0.08^{c}	92.93±0.03^{e}
	US-360-45	91.67±0.09^{e}	92.74±0.19^{e}	93.17±0.24^{d}	94.54±0.31^{f}
	US-360-60	93.15±0.19^{f}	94.24±0.08^{f}	95.06±0.06^{e}	97.81±0.09^{g}
	US-180-30	84.07±0.04^{b}	86.06±0.06^{b}	87.23±0.09^{b}	90.64±0.09^{b}
	US-180-45	86.79±0.04^{c}	88.43±0.04^{c}	90.36±0.09^{c}	91.51±0.04^{cbd}
	US-180-60	88.29±0.11^{d}	89.92±0.05^{d}	91.19±0.10^{c}	92.46±0.06d^{e}
ABTS (%)	Control	0.00±0.00^{a}	68.26±0.18^{a}	70.12±0.16^{a}	73.19±0.11^{a}
	US-360-30	81.96±0.03^{e}	85.55±0.37^{e}	90.32±0.12^{e}	93.23±0.18^{e}
	US-360-45	84.33±0.43^{f}	87.70±0.34^{f}	94.13±0.03^{f}	96.71±0.25^{f}
	US-360-60	89.43±0.28^{g}	93.86±0.03^{g}	96.23±0.18^{g}	98.34±0.27^{g}
	US-180-30	74.63±0.18^{b}	76.41±0.18^{b}	80.71±0.18^{b}	83.61±0.38^{b}
	US-180-45	78.58±0.18^{c}	80.68±0.09^{c}	84.66±0.18^{c}	86.27±0.79^{c}
	US-180-60	80.73±0.03^{d}	83.23±0.18^{d}	86.38±0.52^{d}	89.86±0.09^{d}
Fe^{2+} chelating (%)	Control	0.00±0.00^{a}	46.12±0.11^{a}	50.52±0.22^{a}	53.03±0.51^{a}
	US-360-30	65.85±0.18^{d}	66.67±0.01^{d}	68.56±0.09^{d}	71.53±0.22^{d}
	US-360-45	65.47±0.26^{d}	67.83±0.18^{d}	70.48±0.11^{e}	73.43±0.36^{e}
	US-360-60	68.92±0.05^{e}	71.39±0.55^{e}	74.29±0.26^{f}	76.62±0.49^{f}
	US-180-30	59.76±0.18^{a}	61.14±0.13^{b}	63.59±0.18^{b}	65.27±0.04^{b}
	US-180-45	61.59±0.13^{b}	63.83±0.13^{c}	64.99±0.07^{c}	67.67±0.13^{c}
	US-18-60	63.16±0.11^{c}	66.50±0.66^{d}	67.42±0.49^{d}	70.96±0.04^{d}

Data are presented as mean ± SD of three independent experiments. Different superscript letters within a column indicate significant differences ($p < 0.05$). US; ultrasound

From this study, ultrasound treatments (especially 360W- 60 min) elicited peak accumulations of phenolic acids, flavonoids and anthocyanins, thus accounting for their elevated inhibition of free radicals, and further suggesting that synergistic activity of different phenolic compounds better provide antioxidant capacity, compared to their isolated forms.

Also, the types of individual phenolic compounds elicited with ultrasound treatments, their structure and number of hydroxyl groups are important factors that may have contributed to their elevated antioxidant capacities, compared to phenolic compounds elicited with the control treatments. According to Roginsky and Lissi (2005) phenolic compounds with high number of hydroxyl groups present in their structure exhibit higher hydrogen and electron donations for efficient free radical quenching and antioxidant capacity. Furthermore, it is interesting to highlight that although GPX activity was elevated with ultrasound elicitations (Fig. 5.4 c-d), and signifying the channeling of phenolic compounds for lignification, measured antioxidant capacities were simultaneously higher with ultrasound treatments. Thus, suggesting that ultrasound not only elicited phenolic compounds for structural development and repair against its cavitation effects but also made available free phenolic compounds for efficient antioxidant capacities.

5.4.6 Variations in stress markers and phenolic biosynthesis analyzed by principal component analysis (PCA)

To further understand the effect of ultrasound elicitation and sprouting time on the accumulation of phenolic compounds in common bean sprouts, PCA was applied to all treatments and indicators including stress markers (H_2O_2, CAT and GPX), defense phenylpropanoid triggering enzymes (PAL and TAL) and phenolic compounds (total phenolic acids, flavonoids and anthocyanins) were evaluated. Score plots were used to visualize the relationship between sprouting time and measured indicators, whereas loading plots were used to explain score plots as a function of ultrasound treatments. For PCA analysis on stress markers, all indicators clearly varied from each other, with 100% of this variation explained and captured

on the scatter plot in Fig. 5.6a. The scatter of H_2O_2 and GPX clustered at the positive sides of PC1 and PC2, whereas scores of CAT were scattered at the positive and negative sides of PC1 and PC2, respectively. Importantly, it should also be noted from the scatter plot that activities of CAT and GPX at early sprouting stages were differentiated and not closely clustered to their respective activities at latter sprouting stages. These results correlated with SAS statistical analysis performed in this study, where GPX and CAT were significantly accumulated at latter sprouting stages compared to their levels at early sprouting stages.

From the loading plot (Fig. 5.6 b) it was suggested that, the cluster of H_2O_2 observed on the score plot was due to the close relationship of H_2O_2 accumulated in control treatments along the sprouting process, and not due to H_2O_2 elicitations with ultrasound treatments. Thus, H_2O_2 accumulation in control was not significant over sprouting time, synchronizing with their reported quantitative measurements as discussed above in section 5.4.2.1.1. Also, the cluster of CAT over sprouting time displayed on the score plot was explained from the loading plot to be due to the close relationship in CAT activity elicited with US-180-45 and US-360-30 ultrasound treatments. Also, from the loading plot, it was further suggested that the scatter of GPX on the score plot was due to its varied elicited activities with 180 W (45 min), 180 W (60 min) and 360 W (60 min) ultrasound treatments, with this relationship showing an increasing trend at high ultrasound power and time levels.

As shown on the score plot of Fig. 5.6c, activities of PAL and TAL clearly differed from each other. Secondly, it was clear from PC1 and PC2 that increasing sprouting time contributed to variations in activities of PAL and TAL, with PAL activity being higher compared to TAL. For TAL activity, its scores were mostly scattered at the positive side of PC1.

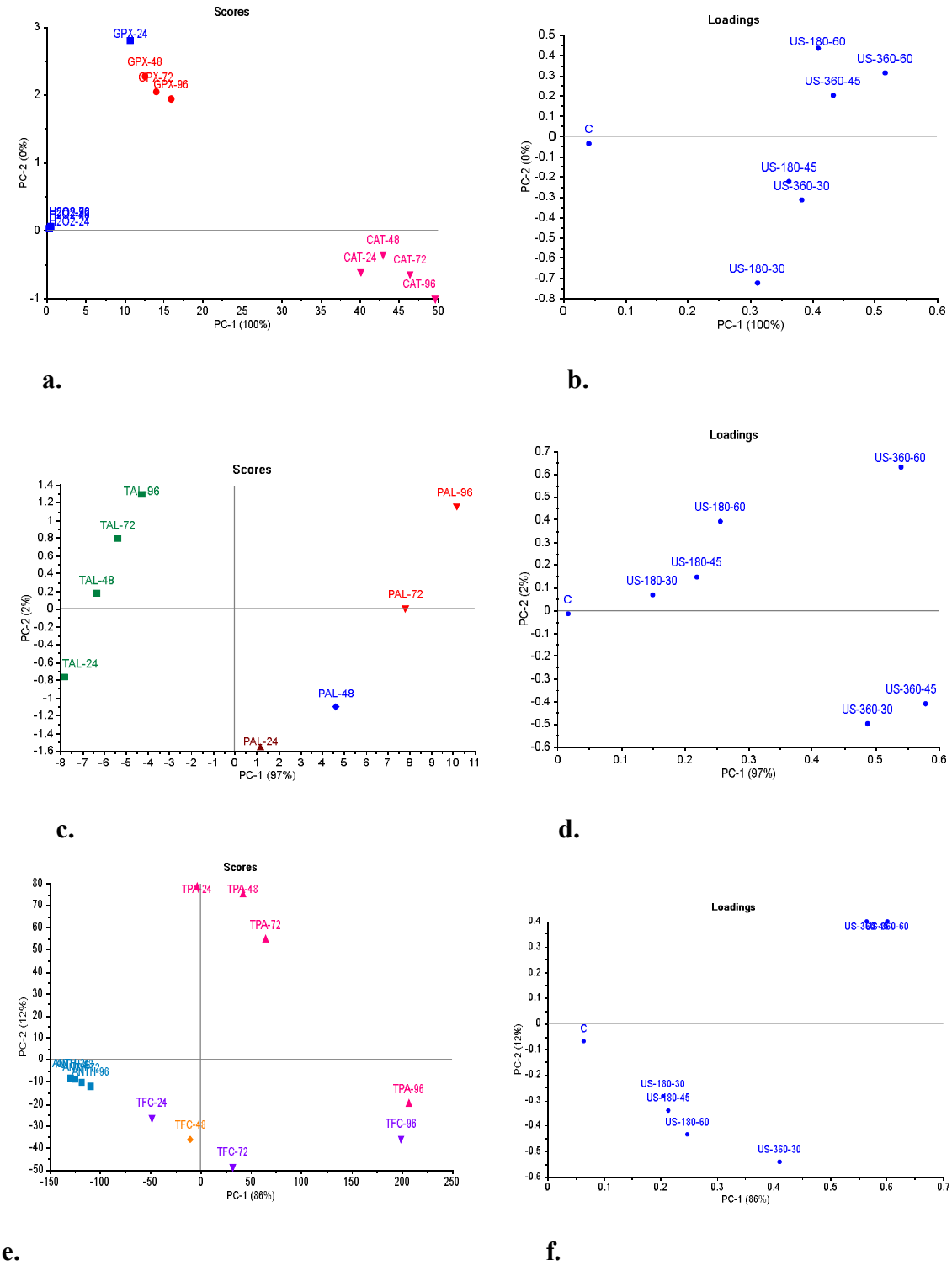


Figure 5.6 (a-f): PCA scores plots and loadings plots of two principal components for stress makers (a and b), defense phenylpropanoid triggering enzymes (c and d) and phenolic compounds (e and f) of common bean sprouts at ultrasound and sprouting stages. US; ultrasound, H_2O_2; hydrogen peroxide, GPX; guaiacol peroxidase, CAT; catalase, PAL; phenylalanine ammonia-lyase, TAL; tyrosine ammonia-lyase, TPA; total phenolic acids, TFC; total flavonoids content, ANTH; anthocyanins

However, scores of PAL activity were divided, with stimulations at the early sprouting times (24 and 48 h) scattered at the positive side of PC1 and stimulations at the latter sprouting times (72 and 96 h) scattered at the positive side of PC2. A further look at the score plots of PAL and TAL indicates a clear negative relationship with respect to sprouting time, strongly suggesting that stimulations of PAL and TAL were significantly varied at each sprouting time.

These results were explained from the loading plot (Fig. 5.6d) where it was suggested that, differences in activities of TAL at sprouting times of 48, 72 and 96 h were mainly due to differences in activities of TAL in response to 180 W (30 min), 180 W (45 min) and 180 W (60 min) ultrasound treatments, respectively. Furthermore, from the loading plot, it was also suggested that, PAL activity at 96 h of sprouting (PAL-96) on the score plot was mainly due to its stimulation with 360 W (60 min) ultrasound treatment and was distinct compared to the other treatments, whereas scores for PAL activity at 24 and 48 h of sprouting (i.e., PAL-24 and PAL-48 h, respectively) on the score plot were suggested to be mainly due to differences in activities of PAL with 360 W (30 min) and 360 W (45 min) ultrasound treatments, respectively, on the loading plot. Thus, the visualized activities of PAL and TAL presented by PCA analysis suggest a relationship between ultrasound and triggering of PAL; TAL at different sprouting stages, with high ultrasound power and time levels significantly influencing PAL, compared to TAL activities. Conclusively, the loading plot differentiated the control from all ultrasonic treatments.

Accumulation of TPA and TFC at 24, 48 and 72 h sprouting times clearly varied from their levels at 96 h, as visualized from the score plot in Fig. 5.6e. However, scatter for both TPA and TFC at 96 h of sprouting clustered closely to each other on the positive side of PC1, suggesting a close relationship in accumulation of these two phenolic compounds at 96 h of sprouting. Interestingly, total anthocyanins (ANTH) at all sprouting times clustered very close to

each other at the negative sides of both PC1 and PC2, signifying a strong relationship in ANTH accumulation over sprouting time. Explaining these results with the loading plot in Fig. 5.6f, ultrasound treatments of 360 W (60 min) and 360 W (45 min) were clearly distinct from other treatments and showed a positive correlation with each other. From the loading plot it can be suggested that, the close relationship visualized with TPA-96 and TFC-96 on the score plot can be correlated with ultrasound treatments of 360 W (60 min) and 360 W (45 min). This result signifies that at 96 h of sprouting, 360 W (60 min) and 360 W (45 min) ultrasound treatments resulted in close accumulations of their respective TPA and TFC contents, which further explains their close independent quantitative results in Table 5.1, where 360 W (60 min) and 360 W (45 min) at 96 h of sprouting reported TPA; TFC contents of 216.74; 203.50 and 196.09; 191.17 mg/100 g, respectively. However, it should be pointed out that, from the loading plot 360 W (60 min) significantly accumulated TPA and TFC, compared to 360 W (45 min) and other treatments. Furthermore, from the loading plot the cluster of ANTH on the scatter plot can be attributed to the close relationship in ANTH accumulations with control treatments as quantitatively depicted in Table 5.1.

5.4.7 Scanning electron microscope analysis

SEM images on the microstructure of cotyledon and seed coat from ultrasound elicited sprouts were assessed and shown in Figures 5.7 - 5.10. For the outer layer studies, SEM images of the cotyledons (Fig. 5.7) showed wounds with ultrasonic treatments, which were otherwise absent on the outer cotyledon surface of the control sprouts at all reported sprouting times.

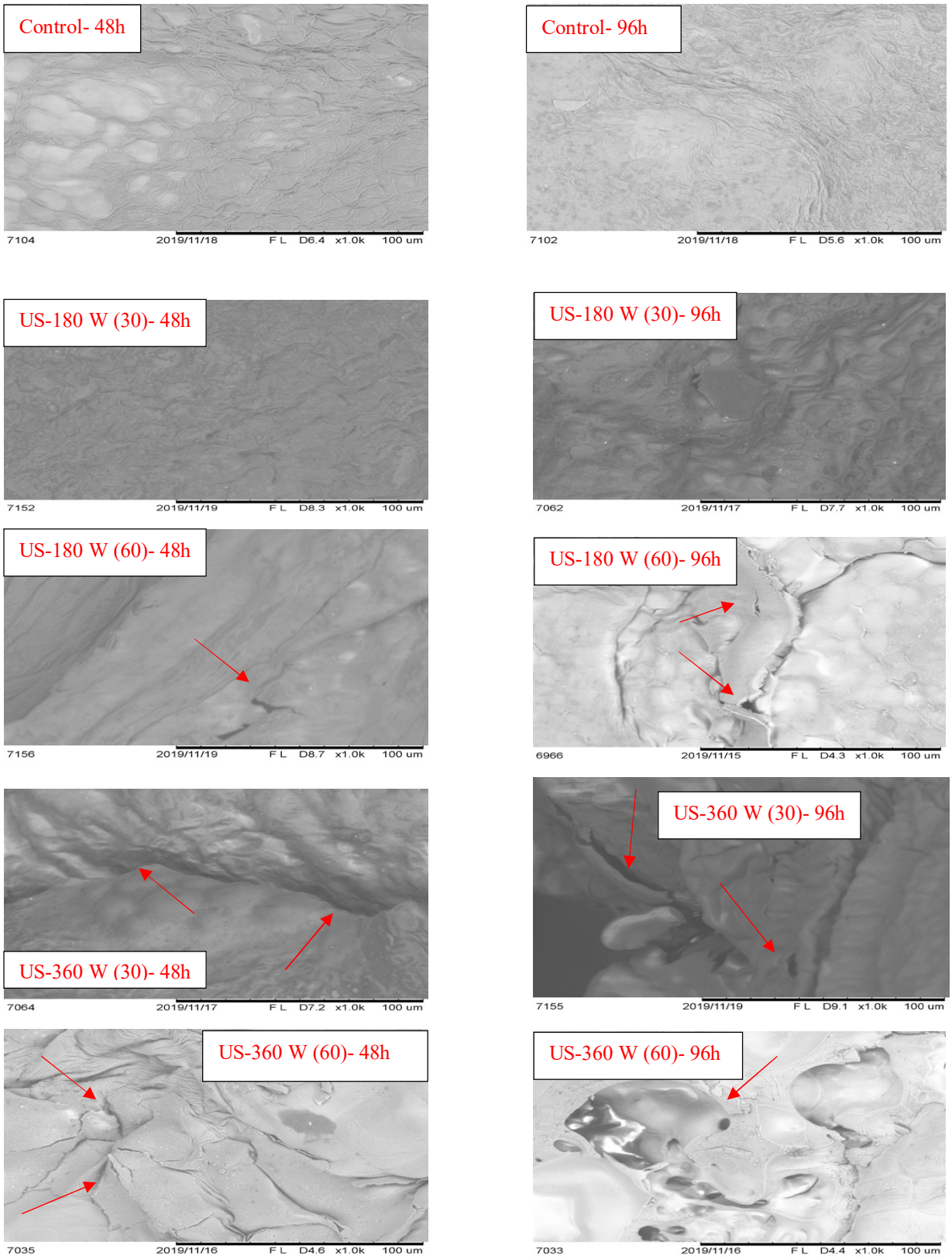


Figure 5.7: Scanning electron micrographs of the outer cotyledon surfaces of ultrasonic treated common bean sprouts. US- Ultrasound

However, for the outer surfaces of the seed coats, wounds were only observed with 360 W (60 min) ultrasonic treatments, compared to the other treatments (Fig. 5.8).

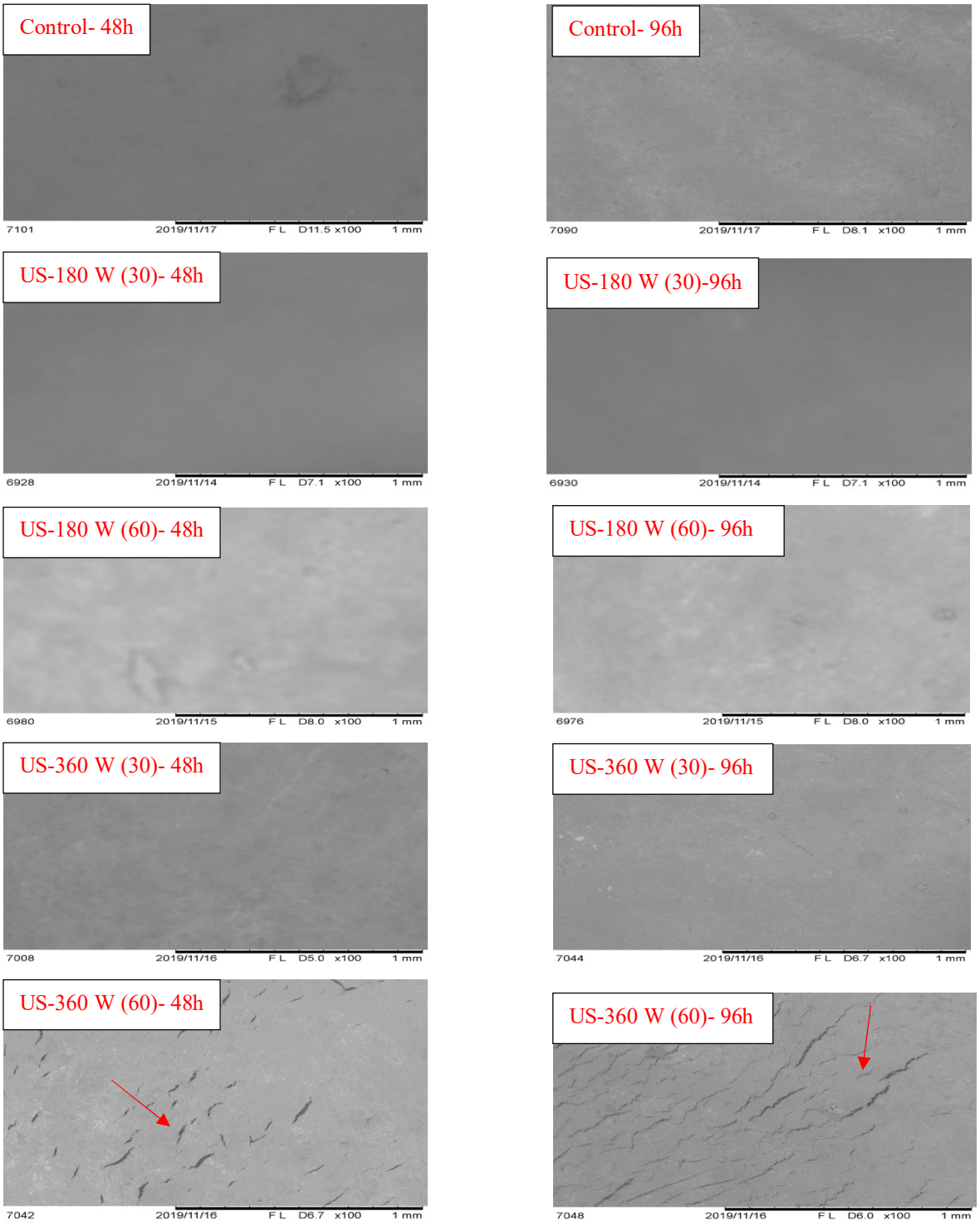


Figure 5.8: Scanning electron micrographs of seed coat surfaces of ultrasonic treated common bean sprouts. US- Ultrasound

This observation can be attributed to the acoustic cavitation induced by ultrasonication, which led to cell wall damage of the seed coat, thus confirming seed coat disruption and the need for phenolic compounds to heal created wounds. Thus, results are in synchrony with the increased activity observed with GPX (the enzyme responsible for mobilization of monophenols to form lignin for healing of damaged cell walls). According to Wang et al. (2019), cavitation created by ultrasonication can cause physical damage and disruption of cell tissues.

Also, SEM images of the inner surfaces of cotyledons showed the presence of pores with ultrasonic treatments compared to the control, with increasing porosity over sprouting time (Fig. 5.9). The pores observed were larger and many, and the structural fragmentation was more pronounced with 360 W (60 min) ultrasound treatments. However, no pores and structural fragmentations were visible with the inner cotyledon SEM images of the control and 180 W (30 min) treatments.

Furthermore, although the seed coat outer surfaces of the 180 W ultrasound treatments showed no wounds, SEM images of its inner surfaces showed defragmentation and small-sized pores (Fig. 5.10). Nevertheless, the largest pores and structural defragmentation were more apparent with the 360 W (60 min) ultrasonic treatment at 96 h of sprouting, compared to its control and 180 W ultrasonic treated counterparts (Fig. 5.10).

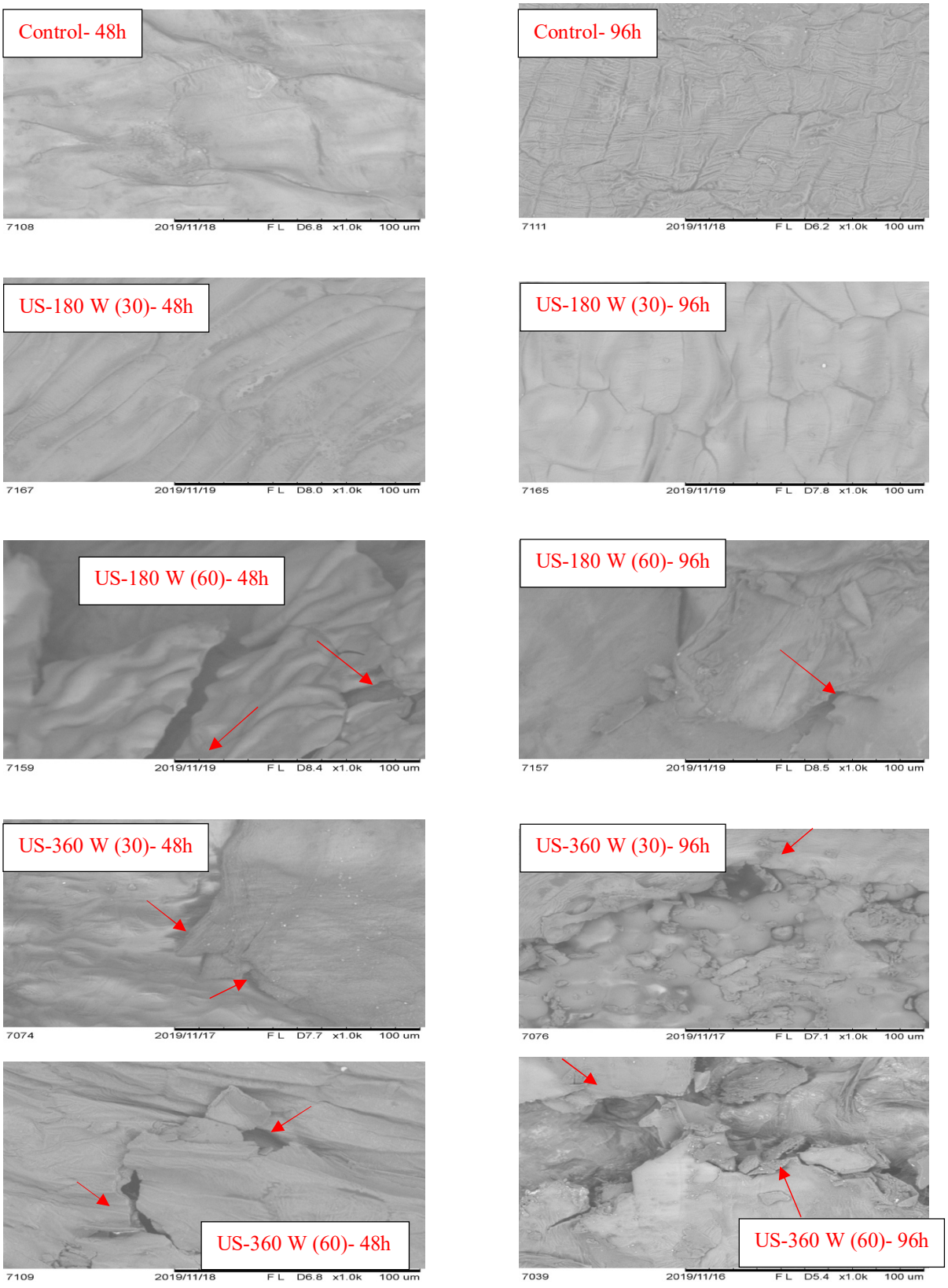


Figure 5.9: Scanning electron micrographs of the inner cotyledon surface of ultrasonic treated common bean sprouts. US- Ultrasound

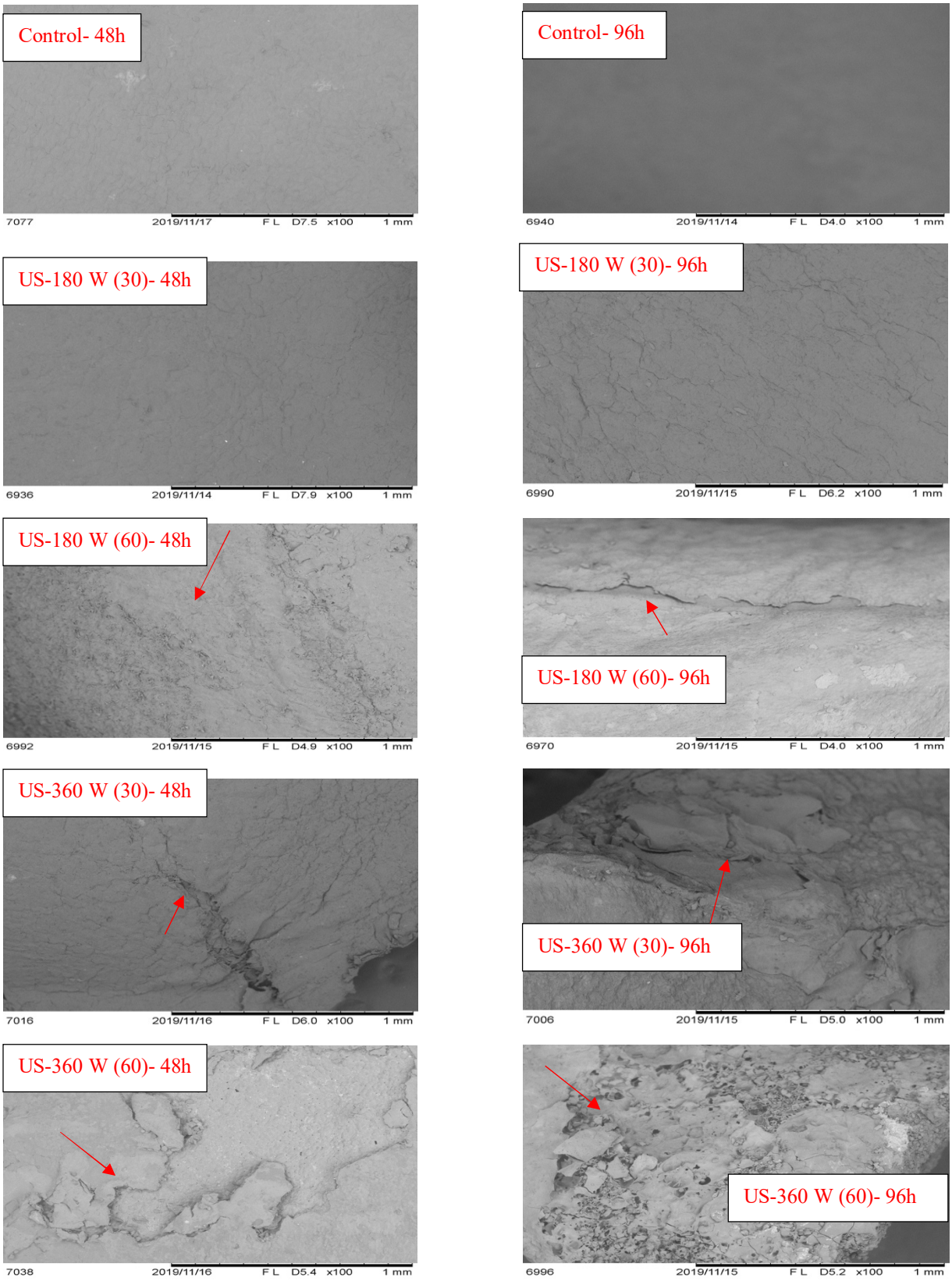


Figure 5.10: Scanning electron micrographs of the inner seed coat layer of ultrasonic treated common bean sprouts. US- Ultrasound

Overall the microstructure studies showed that, increased cavitation at high ultrasound levels contributes to the disruption and loosening of sprout tissues, which was positive for the increased formation of reactive oxygen species and signaling of the phenylpropanoid pathway for enhanced accumulation of phenolic compounds, as reported in this study.

5.5 Conclusion

The major findings of this study are that elicitation with ultrasound at 360 W for 60 min at 96 hr of sprouting significantly increased the biosynthesis of phenolic compounds and antioxidant capacities of common bean sprouts. Stimulation of phenolic compounds correlated with microstructural defragmentation, increased accumulations of H_2O_2, CAT and GPX activities, which further synchronized with the over-expression of PAL and TAL. Furthermore, stimulation of phenolic compounds was linked with enhanced morphological characteristics of sprouts. Additionally, the capacity of ultrasound to improve phenolic accumulation along the sprouting process could be differentiated by their influence in stress accumulation and triggering of PAL and TAL, compared to the control treatments from PCA analysis. Thus, elicitation with ultrasound has potential in the commercial production of phenolic-rich common bean sprouts, with enhanced antioxidative benefits.

CONNECTING STATEMENT TO CHAPTER VI

Results of Chapter V revealed that, high levels of ultrasonication increased structural defragmentation, accumulation of stress markers and stimulation of phenylpropanoid triggering enzymes, leading to the elevated accumulations of phenolic compounds and final antioxidant properties of sprouts, compared to their untreated forms. In chapter VI, production of phenolic compounds and antioxidant properties at different levels of thermal elicitation were investigated during sprouting. Also, kinetic modelling for the production of phenolic compounds during sprouting at different temperatures were evaluated. This chapter has been divided into two manuscripts and they're under review in the Journal of Food and Bioprocess Technology and Journal of Legume Science as follows:

1. Ampofo, J. O., Ngadi, M. and Ramaswamy, H. S. (2020). The impact of high temperature treatments on elicitation of the phenylpropanoid pathway, phenolic accumulations and antioxidative capacities of common bean (*Phaseolus vulgaris*) sprouts. Journal of Food and Bioprocess Technology. (Manuscript number, FABT-D-20-00184).

2. Ampofo, J. O., Ngadi, M. O. and Ramaswamy, H. S. (2019). Elicitation kinetics of phenolics in common bean (*Phaseolus vulgaris*) sprouts by thermal treatments. Legume Science. (Manuscript ID, LEG3-2019-127).

CHAPTER VI

Thermal elicitation of the phenylpropanoid pathway, accumulation of phenolic compounds and antioxidative capacities in common bean (*Phaseolus vulgaris*) sprouts

6.1 Abstract:

Biosynthesis of phenolic compounds in plants increases under abiotic elicitation, such as the application of temperature at different growth stages. However, knowledge on how the induced thermal stress triggers the activity of phenylpropanoid triggering enzymes and accumulation of phenolic compounds during the sprouting stage of pulse foods is limited, especially in common beans. This study was set to investigate how different levels of thermal elicitation (25, 30, 35, and 40 °C) and sprouting time (24, 48, 72 and 96 h) influence the biosynthesis of phenolic compounds and their antioxidative properties in common beans. Oxidative stress factors (H_2O_2, malondialdehyde, catalase and guaiacol peroxidase), activities of phenylpropanoid triggering enzymes (phenylalanine ammonia-lyase and tyrosine ammonia-lyase), phenolic compounds (total phenolic acids, flavonoids and anthocyanins) and antioxidant capacities (DPPH, ABTS and Fe^{2+} scavenging) were monitored with each treatment. Findings showed that, thermal elicitation (especially 30 °C) at 96 h of sprouting significantly increased accumulations of all phenolic compounds and all tested antioxidant properties. This observation corresponded with its maximum activation of phenylpropanoid triggering enzymes throughout the sprouting process, compared to the control (25 °C) and higher temperature levels (35 and 40 °C). Kinetic modelling studies showed maximum activation rates, (k (h^{-1}), of CAT, GPX, PAL and TAL with 30°C treatments. Also, activation energy values for PAL (149 kJ/mol), total phenolic acids (30.4 kJ/mol), and total flavonoids (64.0 kJ/mol) revealed that, they required lower energies for

formations, compared to TAL (221 kJ/mol) and total anthocyanins (209 kJ/mol). Furthermore, the high oxidative stress induced with higher thermal treatments (especially 40 °C) were at lethal levels, thus limiting sprout morphological characteristics, activities of measured antioxidant enzymes, and phenylpropanoid triggering enzymes. Thus, low thermal elicitation of common beans during sprouting can serve as a simple tool for the production of phenolic-rich sprouts.

6.2 Introduction

Common beans (*Phaseolus vulgaris*) belong to a group of food pulses that have been part of the diet of various cultures over the years, with notable examples including kidney, pinto, navy, black, cranberry and red beans. Common beans are advocated as healthy foods by most dietary guidelines due to their rich content of proteins, complex carbohydrates (e.g., resistant starch and dietary fiber), iron and B-vitamins (Gan et al., 2016). However, common beans are healthy not only because of their nutritional value, but also due to the presence of phenolic compounds such as phenolic acids, flavonoids, coumarins and proanthocyanidins (Aquino-Bolaños et al., 2016).

Phenolics are a group of benzene-ringed secondary compounds capable of quenching reactive oxygen species (e.g., H_2O_2, $SO^{\cdot}$, $O^{\cdot}$ etc.,) and provide protection against oxidative stress because of the presence of hydroxyl groups in their structure. They are produced in plants as stress metabolites to defend against biotic and abiotic stress agents. There has been documented evidence on the health benefits of phenolic-rich foods such as antioxidative, anti-inflammatory, antimutagenic, antidiabetic, antibacterial and chemoprotective benefits (Curran, 2012). However, depending on the cultivar, growing conditions, geographical distribution, developmental stage, storage and processing methods, concentration of phenolic compounds varies among common

beans. For instance, Sutivisedsak et al. (2010) reported on navy, pinto, small, black, great northern, pink, light red kidney and dark red kidney beans with total phenolic acids levels of 8.34, 52.9, 10.3, 63.8, 56.5, 56.2 and 60.8 mg GAE/g, respectively. In another study by Aparicio-Fernández et al. (2005) and Akillioglu and Karakaya (2010), black jamapa and pinto bean cultivars were reported to contain total flavonoids levels of 0.69 and 1.27 mg CE/g, respectively. Due to these variations among common beans cultivars, it is of great importance to develop mechanisms based on the biosynthesis principle of phenolic compounds, to help increase their levels, especially in limited cultivars.

One of such an emerging technology is elicitation, which has been proven to elevate plant biosynthesis of secondary metabolites such as phenolic compounds. During this process, the applied stressor/elicitor induces changes in cell structure and anabolism/catabolism of cellular molecules, leading to the reprogramming of plant defense signaling pathways and final accumulation of defense secondary metabolites (Liu et al., 2019). Exposure of plants to thermal stresses leads to the degradation of proteins, lipids and enzymes, as well as loss of the closely packed gel-like plasma membrane into a crystalline flexible plasma membrane (Sita et al., 2017). These associated cell molecular denaturations and loss of membrane integrity results in electrolyte leakages, hormonal imbalance, and increased respiration, leading to an overall elevated concentration of reactive oxygen species (ROS). In response to these generated oxidative stresses, there is an increased signaling of defense mechanisms such as the phenylpropanoid pathway, to increase biosynthesis of phenolic compounds as antioxidants and defense compounds in the plant (Deng and Lu, 2014).

In a previous study, Cáceres et al. (2014) reported that brown rice cultivars sprouted at 28 and 34 °C demonstrated levels of phenolic compounds at 103-307 mg GAE/100 g and 128-260

mg GAE/100 g, respectively, compared to their untreated form (58-78 mg GAE/100 g). Swieca and Baraniak (2014) also reported that sprouting lentils at 40°C elicited total phenolic acids and flavonoids contents of 23.7 and 2.50 mg/g, respectively, compared to their control forms (25°C) which showed 19.8 and 1.84 mg/g contents, respectively.

Nonetheless, although thermal elicitation of sprouts has been shown to be positive, these effects have been assumed to be connected to the generation of reactive oxygen species (ROS) and triggering of the phenylpropanoid pathway, but with limited experimental data to explain this principle, especially with common beans. Thus, the aim of this study was to investigate how thermal elicitation of common beans sprouts will influence biosynthesis of phenolic compounds and antioxidant properties of common bean at different sprouting stages. Results of this study will help design novel systems targeted towards the production of natural phenolic-rich vegetables and ready-to-eat foods, such as edible sprouts.

6.3 Materials and methods

6.3.1 Common bean seeds

Common bean cultivar 'Kabulengeti' was obtained from the Council for Scientific and Industrial Research Center of Zambia. This cultivar has high yield stability; high tolerance to diseases and pests; comprise of protein and lipid contents of 24.4 and 1.38 %, respectively. However, it has low levels of phenolic acids (3.76 mg CE/100 g) and flavonoids (2.24 mg RE/100 g). Therefore, it was chosen for this study as a model for common bean cultivars with low concentrations of phenolic compounds.

6.3.2 Thermal elicited sprouting process

Common bean seeds (100 g) were disinfected with 1% sodium hypochlorite (500 mL) for 20 min and rinsed with distilled water until neutral pH. Next, the seeds were soaked in distilled water (1:3 w/v) at 25ºC for 24 h (Mubarak, 2005). Hydrated seeds were sprouted in darkness at 30, 35 and 40 ºC for 24, 48, 72 and 96 h on petri dishes lined with an absorbent paper in a temperature regulated incubator (Model- 12-140E, Quincy Laboratory, INC. Chicago, U.S.A). During the sprouting process, sprouts were hydrated with distilled water every 6 h. Control sprouting was carried out at 25ºC. After each treatment, the sprouts were collected, measured, washed, frozen, lyophilized and milled using a Cyclone Sample Mill (UDY Corp., Fort Collins, CO, USA) fitted with a 1 mm screen. For each treatment, sprouting was performed in triplicates.

6.3.3 Evaluation of morphological characteristics

6.3.3.1 Physical parameters

Radicle length (cm) of treated sprouts were monitored at each 12 h by measuring from the tip of the radicle to the base of the hypocotyl with a ruler. Hypocotyl diameter (mm) was determined by measuring the hypocotyl width with a Vernier caliper.

6.3.3.2 Sprouting potential

Sprouting percentage, sprouting index and sprout vigor index were measured after every 12 h, and related to sprouting potential (Bormashenko et al., 2012; Ling et al., 2014):

Sprouting percentage (%): $S_p = N_t/N_{TS}$ Eq. (6.1)

Sprouting index: $S_i = \sum N_t/t$ Eq. (6.2)

Sprout vigor index: $S_{vi} = S_i \times L$ Eq. (6.3)

where S_p, S_i *and* S_{vi} are sprouting percentage, sprouting index and sprout vigor index, respectively. N_t is the number of total sprouts showing emerged radicle at time t; N_{TS} is the total number of seeds on the petri dish; *t* is the sprouting time *and L* is the total length of the seedling.

6.3.4 Determination of oxidative stress factors

6.3.4.1 Hydrogen peroxide and lipid peroxidation

Hydrogen peroxide (H_2O_2) was evaluated as described by Alexieva et al. (2001). Approximately 0.1 g of sample flour was homogenized with 1 mL of mixed solution (0.25 mL of 0.1% TCA, 0.5 mL of 1M KI and 0.25 mL of 10 mM potassium phosphate buffer, pH 7.0) at 4°C for 10 min. Afterwards, the obtained mixture was centrifuged (10000 g, 15 min, 4°C), supernatant kept in the dark for 30 min and absorbance measured at 390 nm with a microplate reader. A control was prepared with H_2O instead of KI.

Lipid peroxidation was determined by measuring accumulation of malondialdehyde (MDA) as described by Dhindsa et al. (1981). Approximately, 0.2 g of sample was homogenized in 2 mL of 5% TCA for 10 min and centrifuged at 13500 g for 15 min at 25°C. Afterwards, 1 mL of supernatant was mixed with 1 mL of 0.5% (v/v) thiobarbituric acid (in 20% (v/v) TCA). The obtained mixture was heated at 96°C for 30 min, cooled in an ice bath and centrifuged at 9500 g for 10 min. MDA content was expressed as nmol MDA per g of dry weight.

6.3.4.2 Determination of antioxidant enzymes

6.3.4.2.1 Extract preparations and protein content determination

6.3.4.2.2 Total protein assay

Protein contents of enzyme extracts were measured as described by Bradford (1976). Bradford reagent was prepared by diluting the dye concentrate in a 1:4 ratio with distilled water. To 20 µL of enzyme extract, 1 mL of the prepared Bradford reagent was added, incubated at room temperature for 5 min, vortexed for 5 sec, and absorbance measured at 595 nm using a microplate reader. For the control, enzyme extract was replaced with extraction buffer and subjected to the same conditions as described above. Protein assay was performed on the same day of extraction.

6.3.4.2.3 Enzyme extraction

Briefly, 200 mg of sample was homogenized at 4°C for 20 min in 4 mL of sodium phosphate buffer (100 mM, pH 6.4) containing 0.2 g of polyvinylpyrrolidone. Next, the homogenized mixture was centrifuged (12000 g, 4°C, 30 min), and the supernatant collected as catalase and guaiacol peroxidase extracts (Swieca et al., 2014). Catalase (CAT) and guaiacol peroxidase (GPX) assays were performed on the same day of extraction. Enzyme extractions were performed in triplicate.

6.3.4.2.4 Enzymatic assays

For CAT activity, 0.05 mL of enzyme extract was mixed with 0.95 mL of H_2O_2 (10 mM in 100 mM sodium phosphate buffer, pH 7) and incubated for 1 min at 30°C. Decomposition of H_2O_2 was measured at 240 nm with a microplate reader. One unit (1U) of CAT activity was

defined as the amount of CAT capable of decomposing 1.0 µmol H_2O_2 per min under the conditions of the assay. Results were expressed as U/mg protein (Swieca et al., 2014).

Guaiacol peroxidase (GPX) assay was performed as described by Burguieres et al. (2007). Enzyme extract (0.1 mL) was incubated with 2 mL guaiacol (8 mM, in 100 mM sodium phosphate buffer, pH 6.4) for 1 min at 30°C. Afterwards, 1 mL of H_2O_2 (24 mM) was added and the change in absorbance was measured at 460 nm with a microplate reader. GPX activity was expressed as U/mg protein, where 1U = 0.001 Δ460/min under the conditions of the assay.

6.3.5 Evaluation of defense phenylpropanoid triggering enzymes

6.3.5.1 Enzyme extraction

Two hundred (200) mg of sample flour was homogenized with 2 mL extracting buffer (0.2 M boric acid buffer containing, 1 mM EDTA and 50 mM β-mercaptoethanol, pH 8.8) for 20 min at 4°C. Afterwards, the homogenized mixture was centrifuged (12000 g, 4°C, 30 min) and supernatant collected as enzyme extract (Swieca et al., 2014). Enzymatic extractions were performed in triplicates.

6.3.5.2 Enzymatic assays

Assay of phenylalanine ammonia-lyase (PAL) was evaluated by incubating 300 µL enzyme extract, 1.2 mL of 0.02 M L-phenylalanine and 2 mL extracting buffer at 30°C for 60 min. After incubation, reaction was terminated by adding 0.5 mL of 10% TCA, centrifuged (15000 g, 10 min, 4°C) and absorbance of the supernatant measured at 290 nm with a microplate reader. One unit (1U) was defined as the amount of PAL that produced 1.0 µmol trans-cinnamic

acid per min under conditions of the assay. Results were expressed as U/mg protein (Assis et al., 2001).

Assay of tyrosine ammonia-lyase (TAL) was determined by incubating 100 µL enzyme extract and 0.9 mL of 0.02 M L-tyrosine at 30°C for 60 min. Next, the reaction was stopped by adding 0.5 mL of 10% TCA, centrifuged (15000 g, 10 min, 4°C) and absorbance of the supernatant read at 310 nm with a microplate reader. One unit (1U) was defined as the amount of TAL that produced 1.0 µmol *p*-coumaric acid per min under conditions of the assay. Results were expressed as U/mg protein (Assis et al., 2001).

6.3.6 Assessment of phenolic compounds and antioxidant capacities

6.3.6.1 Extraction of phenolic compounds

Phenolics were extracted as described by Marathe et al. (2011). About 1 g of sample flour was extracted with 15 mL of 80% aqueous methanol (V/V), centrifuged (5000 rpm, 10 min), and supernatant filtered. Afterwards, methanol in the supernatant was evaporated using a rotary evaporator and obtained phenolic compound extracts were freeze-dried for subsequent analysis.

6.3.6.2 Determination of total phenolic acids and anthocyanins

Total phenolic acids and anthocyanins content of methanolic extract were determined according to the method of Mazza et al. (1999). A 20 µL methanolic extract was mixed with 240 µL of 2% HCL in 75% methanol, vortexed for 1 min and absorbances at 280 and 520 nm read with a microplate reader for total phenolic acids and anthocyanins, respectively. Standard curves were prepared under similar conditions with (+)-catechin (0-200 $\mu g mL^{-1}$) and cyanidin-3-

glucoside (0-20 μgmL^{-1}) for quantitative measurements of total phenolic acids and anthocyanins, respectively.

6.3.6.3 Determination of total flavonoids

Total flavonoids content was analysed as described by Hairi et al. (1991). Exactly 50 µL of methanolic extract was mixed with 200 µL of 10 gL^{-1} 2-aminoethyldiphenylborate, vortexed for 1 min, and absorbance measured at 404 nm with a microplate reader. A standard curve for quantitative measurement was prepared under the same condition with rutin (0-50 μgmL^{-1}).

6.3.6.4 Antioxidant activities

6.3.6.4.1 Estimation of DPPH free radical scavenging capacity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging capacity was analysed as described by Akond et al. (2011). Briefly, 83 µL sample phenolic compound extract was mixed with 167 µL of 0.1 mM DPPH free radical solution, vortexed for 30 sec, kept in the dark for 30 min, and absorbance measured with a microplate reader at 517 nm. A control reaction mixture was prepared by replacing the phenolic compound extract with 80% methanol under the same conditions. Ability of the phenolic compound extract to scavenge DPPH free radical was determined using Eq. 6.4 below:

$$\% \text{ Scavenging activity} = \frac{A_{control} - A_{test}}{A_{control}} \times 100 \qquad \text{Eq. (6.4)}$$

6.3.6.4.2 Estimation of ABTS scavenging capacity

ABTS scavenging capacity was determined using the method of Re et al. (1999). ABTS free radical solution was prepared by mixing ABTS (14 mM, 5 mL) and potassium persulfate (4.9 mM, 5 mL) solutions and storing in the dark for 16 h at 25°C. Before using this solution, it was diluted with distilled water to get an absorbance of 0.900 ± 0.020 at 734 nm. Final reaction mixture comprised of 200 µL ABTS free radical solution and 50 µL sample phenolic compound extract, vortexed for 1 min, kept in the dark for 6 min and absorbance measured at 734 nm with a microplate reader. Control reaction mixture was prepared by replacing phenolic compound extract with 80% methanol under similar conditions of the assay. Ability of the phenolic compound extract to scavenge ABTS free radical was determined using Eq. 6.4.

6.3.6.4.3 Estimation of ferrous (Fe^{2+}) chelating capacity

Capacity of sample phenolic extracts to chelate ferrous ions was estimated by the method of Dinis et al. (1994). A 50 µL sample phenolic extract was added to Fe (II) chloride (0.002 M, 25 µL) and ferrozine (0.005 M, 200 µL), and incubated in darkness for 10 min at 25°C. Afterwards, absorbance of the reaction mixture was measured at 562 nm with a microplate reader. Chelating capacity of sample phenolic extract was determined using Eq. 6.4.

6.3.7 Kinetic analysis

According to Li et al. (2018) and Oancea et al. (2018) the first order kinetic model is suitable for studying activities of enzymes and accumulation of biochemical components. Thus, kinetics of all investigated biomolecules, enzymes and antioxidant capacities were calculated according to the following first order equation:

$$\ln(A) = -Kt + \ln A_o \quad \text{Eq. (6.5)}$$

Furthermore, the Arrhenius equation was used to evaluate the temperature dependence of the reaction rate *k*:

$$k = k_o exp^{\left(\frac{-E_a}{RT}\right)} \quad \text{Eq. (6.6)}$$

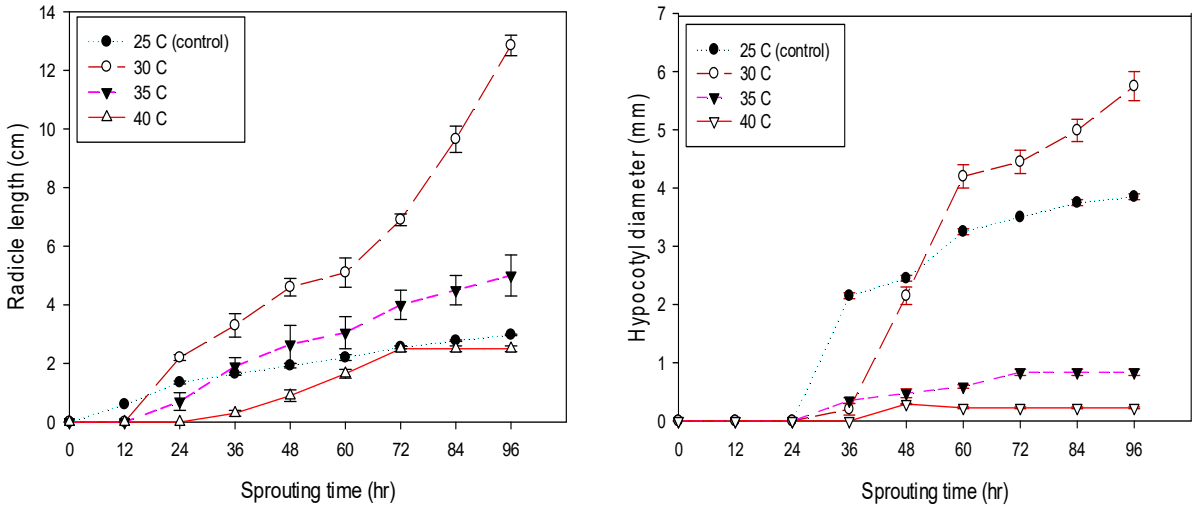
where k_O is the pre-exponential factor h^{-1}, E_a is the activation energy of the enzymatic reaction; *R* is the gas constant; and *T* is the absolute temperature.

6.4 Results and discussion

6.4.1 Effect of temperature treatments on morphological characteristics of common bean sprouts

6.4.1.1 Radicle elongation and hypocotyl diameter

Generally, radicle and hypocotyl growths of common bean sprouts were very responsive to all temperature treatments. Although the thermal treatments seemed to delay radicle emergence at the first (12 h treatment, Fig. 6.1a) compared to the control, there was a change when the treatment was extended to 24 h of sprouting. Overall, the longest radicle length of 12.9 cm was elicited with 30°C at 96 h of sprouting. This length was 77, 56 and 80 % longer than the control, 35, and 40 °C treatments at the same sprouting time.



a. **b.**

Figure 6.1(a-b): Effect of temperature treatments on radicle (a) and hypocotyl (b) growths of common bean sprouts. Results are presented as mean ± SE

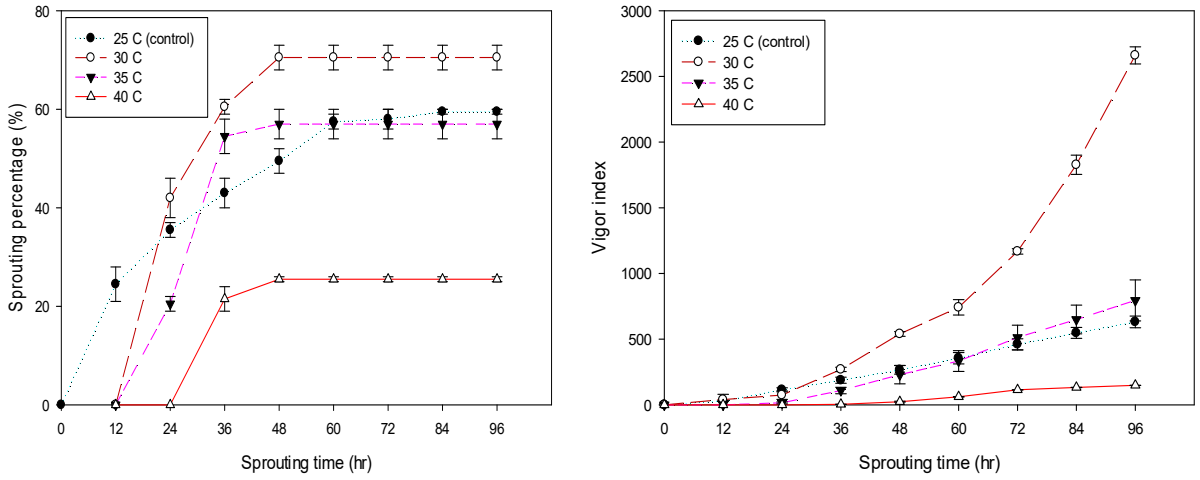
Radicle growth was significantly limited with 40°C treatments throughout the sprouting process. High temperature stress has been reported to arrest hormones and enzymes involved in normal metabolic processes of biological systems (Phan et al., 2013). Thus, the reduced radicle growth observed with temperature treatments in this study could be attributed to the limited expression of auxin (the main hormone responsible for radicle elongation) at the radicle tip (Simon and Petrášek, 2011), as well as the possible denaturation of endogenous nutrients needed as driving forces for efficient division of apical meristems localized in the radicle.

Figure 6.1 b demonstrates that all thermally stressed sprouting treatments inhibited hypocotyl emergence from the seed coat from the onset of sprouting until 36 h while the control showed an emerged hypocotyl with a diameter of 2.15 mm, at the same sprouting time. However, continuous elicitation up to 60 h showed a different trend, when the highest hypocotyl diameter was elicited at 30°C. Under this condition, the hypocotyl diameter was 23, 86 and 95% higher than the control, 35 and 40 °C treatments, respectively, and continued further as the

sprouting process was prolonged. This result could be attributed to the capacity of 30°C to enhance hormonal and hypocotyl apical meristematic activities along the sprouting process. However, it should be noted that sprouting at 35 and 40 °C reduced hypocotyl growths by 78 and 94 %, respectively, compared to the control at 96 h of sprouting.

6.4.1.2 Sprouting percentage and vigor index

As presented in Fig. 6.2a-b, elicitation at 30°C significantly enhanced sprouting percentage and vigor index of common beans compared to the other thermal treatments. Corresponding to radicle growth, except for the control, all treated samples contributed to delaying the capacity of sprouting during the first 12 h. However, further exposure to the thermal stresses resulted in enhanced sprouting percentages, significantly at 30°C, as compared to the control. Overall, the highest sprouting percentage of 71% was obtained at 30°C at 48 h of sprouting, with no further increase up to 96 h of sprouting. This sprouting percentage was 1.42, 1.24 and 3.08 folds higher than observed with the control, 35 and 40 °C treatments, respectively. It should also be noted that, the sprouting percentage observed at 35°C was comparable to the control from 60-96 h of sprouting, whereas elicitation at 40°C significantly reduced sprouting percentage throughout the sprouting process.



a. **b.**

Figure 6.2(a-b): Effect of temperature treatments on sprouting percentage (a) and vigor index (b) of common bean sprouts. Results are presented as mean ± SD

This result may be due to moisture evaporation, hormones and enzymes inactivation, increased respiration and low nutrient availability to drive physiological processes at high temperature levels. These results are in agreement with the work of Gairola et al. (2011) who observed 8.22 and 22.1 % decrease in sprouting percentages at 37 and 35°C of *Jatropha curcas*, compared to its 30°C treatments. Also, Islam et al. (2017) reported higher sprouting percentage at 30°C for lignosus bean, compared to 25°C. According to Ruan (2014) scientific works on non-pulse crops have attributed reduced sprouting percentages with high temperature stress to low moisture content, increased respiration and sugar depletion. For instance, Ahmed et al. (2015), observed decreased activities of hydrolytic enzymes involved in endospermic starch and protein metabolism at the early developmental stages of rice, which limited ATP and amino acids availabilities for efficient embryonic growth.

Furthermore, since the current study defined sprouting percentage as the number of bean seeds with emerged radicles from the seed coat, results of sprouting percentage was in synchrony with results of radicle growth where 30°C elicited the highest radicle length at 48 has observed

with sprouting percentage. Another significant observation to be pointed out is that, the treated samples showed no sprouting increases after 48 h. This may infer that the capacity of a common bean seed to mobilize endogenous factors needed to break dormancy occurs at the early stages of sprouting when subjected to thermal elicitation.

Additionally, vigor index of common beans was delayed with all treated samples until 36 h of sprouting where bean seeds begun to positively respond to thermal treatments (Fig. 6.2b). After 96 h of sprouting, the maximum vigor index was elicited with 30°C treatment. This elicited vigor index was significantly higher than observed with the control, 35 and 40 °C treatments by 77, 70 and 94 %, respectively. Compared to the control, the ability of common bean seeds to overcome dormancy, efficiently sprout and develop its morphological features at 30°C appear to confirm an inherent ability of common beans to sprouts under low tropical and subtropical conditions. Similar to sprouting percentage, vigor index of sprouts elicited with 35°C was parallel to control, whereas elicitation with 40°C significantly reduced vigor index over that of the control (77%) and other treated samples.

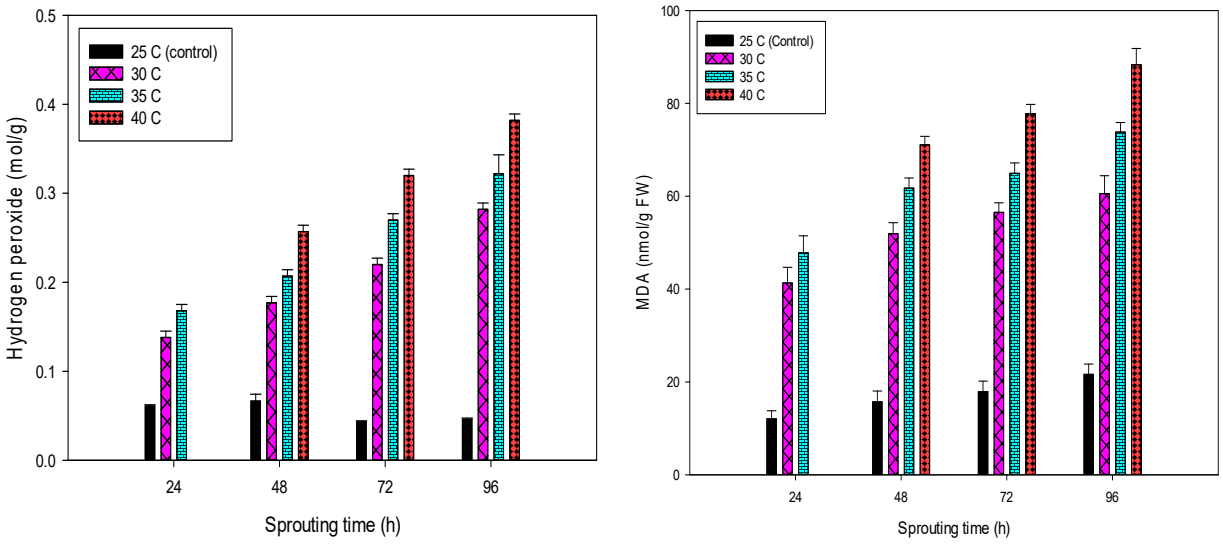
6.4.2 Characterization of oxidative stress markers

6.4.2.1 Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) accumulations

Exposure of plant cells to temperature beyond their optimum thresholds induces damage to cellular molecules and increases respiratory activities, which may result in negative effects when end-metabolites such as reactive oxygen species (e.g., H_2O_2) are accumulated above threshold levels (Kumar et al., 2013). From Fig. 6.3a it was clear that thermal elicitation of common bean sprouts significantly ($P < 0.05$) elevated H_2O_2 contents compared to the control, with this effect increasing at higher temperature and sprouting time levels. The highest level of

H_2O_2 was elicited with 40°C at 96 h, and this was 1.3, 1.18 and 8.3 times higher than that of the 35, 30 °C and control treatments, respectively. This result further explains reduced morphological characteristics observed with 40°C treatments suggesting that the 40°C sprouting resulted in toxic oxidative stress due to accumulation of H_2O_2 that was beyond sprout threshold levels and capable of limiting embryonic growth and development during sprouting.

High temperature stress in plants has been linked to lethal levels of H_2O_2, capable of arresting physiological processes by inducing oxidative damages to proteins, hormones, enzymes and lipids (Nahar et al., 2015). However, it should also be noted that although 30 and 35 °C treatments also elicited higher H_2O_2 levels compared to the control, it did not negatively influence measured morphological characteristics, especially at 30°C. According to Cavusoglu and Kabar (2010) threshold levels of H_2O_2 enhance sprouting by helping break seed dormancy and promoting hormonal balance associated with the sprouting process.



a. **b.**

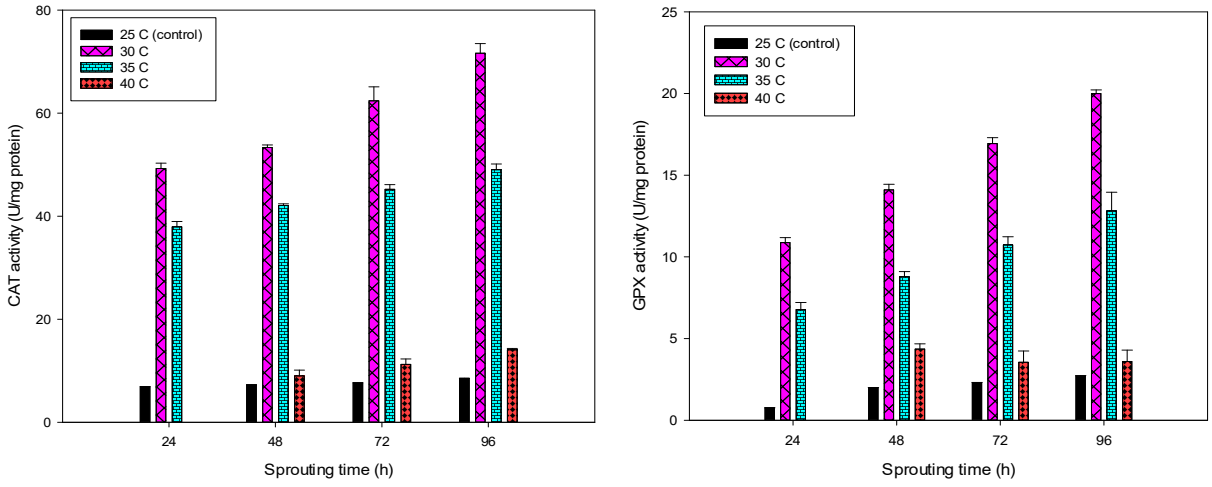
Figure 6.3(a-b): Effect of temperature treatments on hydrogen peroxide (a), and MDA (b) contents of common bean sprouts. Malondialdehyde (MDA). Results are presented as mean ± SD

Parallel to H_2O_2, concentrations of malondialdehyde (MDA) increased significantly ($P < 0.05$) with temperature rise and sprouting time (Fig. 6.3b). MDA is a product of lipid peroxidation, with its presence signifying oxidative stress and damage to lipids in cell membranes (Swieca et al., 2014). Overall, MDA was significantly higher in 40°C elicited sprouts at 96 h of sprouting (an increase of about 16.4, 31.4 and 75.1 % in comparison to their 35, 30 °C and control counterparts, respectively). MDA concentrations observed with 40°C further explain their reported elevated levels of H_2O_2 and reduced morphological characteristics (Mishra and Agrawal, 2014). Also, excess levels of MDA at 40°C signify higher plasma membrane damage and possible leakage of nutrients and growth factors from sprout cytosol (Alexia et al., 2014), thus, reducing available nutrients, hormones and other growth factors required for efficient physiological processes and morphological growth. However, it should also be noted that, although MDA accumulations were higher with 30 and 35 °C treatments, they did not negatively influence their morphological characteristics, especially with 30°C.

6.4.2.2 Antioxidant enzymes

Stimulation of oxidative stress was also confirmed by elevated activities of CAT and GPX enzymes. Relative to 35, 40 °C and control, elicited CAT activity was 32, 80 and 88 % higher with 30°C at 96 h of sprouting (Fig. 6.4a). This observation could also account for their enhanced morphological characteristics measured in this study, as CAT has been demonstrated to protect cells by decomposing accumulated H_2O_2 into oxygen and water. According to Gupta et al. (2013) sprouting under optimal temperatures elevates activities of endogenous antioxidant enzymes such as CAT to balance and enhance redox homeostasis. It should also be mentioned that, although oxidative stress (H_2O_2 and MDA levels) accrued with increasing temperature

levels in this study, this trend was not fully reflected with CAT response. This result may be due to enzyme tertiary structure and substrate denaturations at high levels of H_2O_2 and MDA. For example, temperature stress at 38°C with soybean was shown to result in 44.6% decrease in CAT activity, leading to increased accumulations of H_2O_2 (70.4%) and plasma membrane damage (54.7%) over that of the control treatments (28°C) (Djanaguiraman and Prasad, 2010).



a. **b.**

Figure 6.4(a-b): Effect of temperature treatments on CAT (a) and GPX (b) activities of common bean sprouts. Catalase (CAT), Guaiacol peroxidase (GPX). Results are presented as mean ± SD

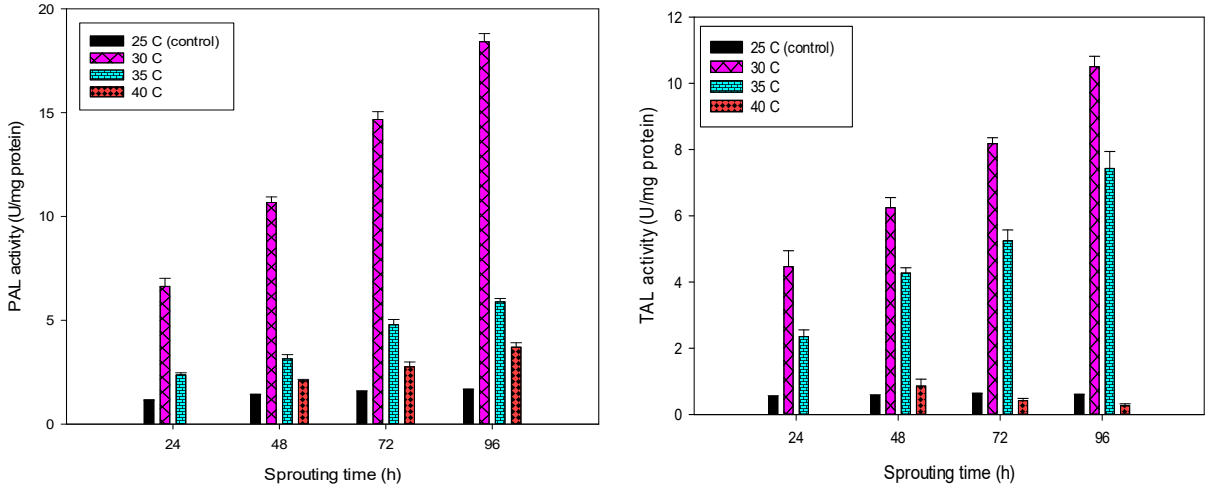
Similar to CAT, activity of GPX also increased with sprouting time, with this drift decreasing with increasing sprouting temperature (Fig. 6.4b). Overall, the greatest GPX activity of 19.40 U/mg protein was ($P < 0.05$) elicited with 30°C at 96 h of sprouting, with this observation being 1.58, 4.77 and 7.24 folds higher, relative to the 35, 40 °C and control treatments, respectively. GPX is the enzyme responsible for lignification towards structural development of the cell wall (Erofeeva, 2015). Its elevated activity at 30°C signifies high protection against accumulated H_2O_2 and MDA, thus inferring reduced plasma membrane damage and reduced leakage of nutrients out of sprout cytosol, as compared to the control and

higher temperature treatments. Therefore, this study suggests that thermal elicitation of common beans sprouts at 30°C can induce oxidative stress at threshold levels that can stimulate higher activities of CAT and GPX for enhanced structural development and morphological growth.

6.4.3 Defense phenylpropanoid triggering enzymes

6.4.3.1 Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities

Under stress, one mechanism by which plants defend themselves is to trigger the phenylpropanoid pathway, the main pathway responsible for biosynthesis of defense phenolic compounds. As graphically presented in Fig. 6.5a-b, thermal treatments significantly ($P < 0.05$) elevated activities of PAL and TAL (mostly with PAL compared to TAL), with the trend decreasing at high temperature levels. After 96 h of sprouting, the optimal PAL activity of 18.41 U/mg protein was elicited with 30°C, with this observation being 3.12, 4.96 and 11.0 folds higher, relative to their 35, 40 °C and control counterparts, respectively.



a. **b.**

Figure 6.5(a-b): Effect of temperature treatments on PAL (a) and TAL (b) activities of common bean sprouts. Phenylalanine ammonia-lyase (PAL), Tyrosine ammonia-lyase (TAL). Results are presented as mean ± SD

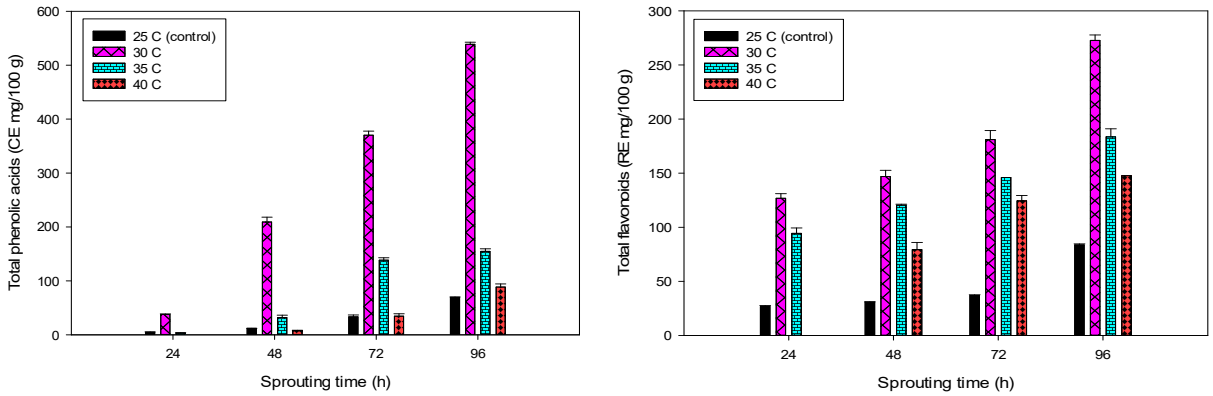
Equivalent to PAL activity, 29, 94 and 97 % increases of TAL activity were observed with 30 °C elicitations, over that observed with 35°C, control and 40°C treatments, respectively. According to He et al. (2011), stress accumulations during sprouting induces optimum expression of enzymes involved with the phenylpropanoid pathway. Reduced activities of PAL and TAL at high temperature levels, especially with 40°C can be attributed to the excessive accumulations of H_2O_2 and MDA beyond sprout threshold levels, which may have damaged the functional tertiary structure of PAL and TAL, as well as possible denaturations of their proteinaceous substrates (Liu et al., 2019). To the best of our knowledge, the current study is the first work to investigate how different levels of thermally induced oxidative stresses triggered PAL and TAL activities during the sprouting process of common beans. In agreement with the current study is the work of Eissa and Ibrahim (2018), who screened activities of crude PAL and TAL at different temperatures. These authors reported optimal relative activity of PAL (100-94 %) and TAL (100-90 %) at 30°C, compared to their observed activities at 40°C (PAL; 68-56 %, TAL; 55-45 %) and 50°C (PAL; 54-48 %; TAL; 53-43 %), relating their observations to the reports of Andrea and Christine (2013) and Duangmal and Apenten (1999), that high temperature stress induces heat denaturations of PAL and TAL functional tertiary structures.

6.4.4 Accumulations of phenolic compounds and antioxidant properties

6.4.4.1 Total phenolic acids, flavonoids and anthocyanins

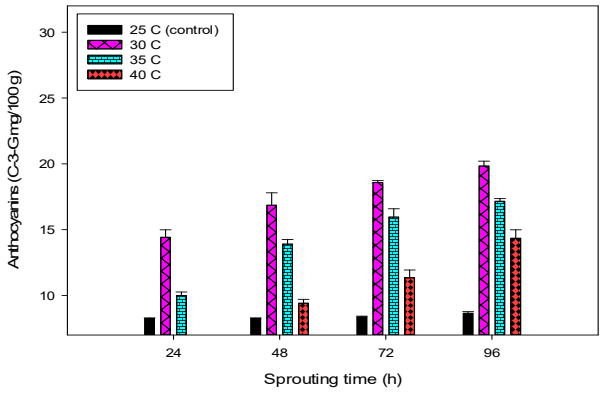
Due to the fact that oxidative stress stimulates the biosynthesis of phenolic compounds as defense agents, this study investigated accumulation of phenolic compounds during thermal elicitation of common bean sprouts. Fig. 6.6a-c demonstrates the thermal elicitations of phenolic compounds, which increased at relatively lower temperatures and high sprouting time. Overall,

all thermal treatments elevated levels of phenolic acids, flavonoids and anthocyanins as compared to the control. Concentrations of total phenolic acids (Fig. 6.6a) was maximally elicited with 30°C at 96 h of sprouting, being 3.49, 6.08 and 7.75 times higher, relative to their 35, 40 °C and control counterparts, respectively. Similarly, the highest level of total flavonoids (155 mg rutin/100 g) was also elicited under the same condition, and was higher compared to the 35, 40 °C, and control treatments by 33, 46 and 69 %, respectively. Parallel to total phenolic acids and flavonoids, 30°C at 96 h of sprouting was also optimal for the accrual of total anthocyanins, by being 1.16, 1.38 and 2.29 folds, compared to the 35, 40 °C, and control treatments, respectively.



a.

b.



c.

Figure 6.6(a-c): Effect of temperature treatments on total phenolic acids (a), total flavonoids (b), and anthocyanins (c) contents of common bean sprouts. Results are presented as mean ± SD

The maximum build-up of phenolic compounds elicited with 30°C could be explained by the significant activities of PAL and TAL reported earlier. However, it should be noted that although high temperature levels, especially 40°C, were reported to increase accumulations of H_2O_2 and MDA, this did not reflect in their capacity to trigger activities of PAL and TAL, due to the possible negative effects of these oxidative molecules against enzyme structure and final catalysis (Yang et al., 2018). Thus, high temperature treatments (35 and 40 °C) resulted in reduced accumulations of phenolic compounds.

Furthermore, it is also important to note that, enhanced build-up of phenolic compounds with 30°C correlated with improved morphological characteristics of common bean sprouts, hence further confirming the defense link between phenolic compounds and sprout growth as reported by Burguieres et al. (2007). Considering the demand for foods that are beyond basic nutrition but also offering additional health benefits, findings from this study suggest that sprouting at 30°C can elevate expressions of phenylpropanoid triggering enzymes, for final maximization of phenolic compounds. Similar to this study, Cáceres et al. (2014) reported on elevated total phenolic contents of 5 rice cultivars sprouted at 28°C for 96 hr, compared to their higher temperature counterpart (34°C) at the same sprouting time, explaining their observation to the possible inductions of PAL and TAL.

6.4.4.2 Evaluation of antioxidant capacities

Bioactivity of phenolic compounds are essential to their health claims, thus, this study further investigated antioxidant properties of the phenolic compound extracts obtained from thermally treated common bean sprouts. From Table 6.1, results of this study show that thermal elicitation significantly ($P < 0.05$) enhanced antioxidant potential of common bean sprouts, and

this enhancement increased with longer sprouting time. DPPH scavenging values were up to 3.04, 5.01 and 6.52 % higher after eliciting common bean sprouts at 30°C for 96 h, as compared to 35, 40 °C and control treatments, respectively, at the same sprouting time. Comparable to DPPH scavenging, ability to quench ABTS free radicals was also optimally elicited with the 30°C treatment (96%) at 96 h of sprouting, with this being 3.1, 7.8 and 15 % higher compared to the 35, 40 °C and control treatments, respectively. Similarly, capacity of phenolic compound extracts to chelate ferrous ions was also reported to be highest (68.28%) with 30 °C, and this was 2.0, 5.2 and 7.0 % higher, compared to 35°C, 40 °C and control treatments, respectively.

Therefore, common beans sprouted at 30°C for 96 h elicited phenolic acids, flavonoids and anthocyanins, with the highest free radical chelating capacities. This trend can thus be linked to their increased and synergistic phenolic compound compositions. This observation could also be due to the capacity of sprouting at 30°C to elicit particular groups of phenolic compounds with higher hydroxyl groups and lower glycosylation, than the other investigated temperature levels.

Conclusively, this study showed that 30°C elicitation of common beans along the sprouting process can induce stress at threshold non-toxic levels. Therefore, elevating the phenylpropanoid pathway towards the development of sprouts that will significantly improve the antioxidant status of consumers.

Table 6.1: Antioxidant capacities of phenolic compounds extracts from thermal elicited common bean sprouts

Antioxidant method	Treatment	Sprouting time (h)			
		24 h	48 h	72 h	96 h
DPPH (%)	Control (25 °C)	77.89±0.81^{b}	81.24±0.51^{a}	83.82±0.51^{a}	88.03±0.62^{a}
	30 °C	88.79±0.09^{d}	90.46±0.33^{d}	92.65±0.11^{d}	94.17±0.11^{c}
	35 °C	86.82±0.00^{c}	87.65±0.11^{c}	89.04±0.22^{c}	91.23±0.33^{b}
	40 °C	0.00±0.00^{a}	84.75±0.11^{b}	86.30±0.22^{b}	89.40±0.21^{a}
ABTS (%)	Control (25 °C)	70.67±0.05^{b}	72.31±0.31^{a}	74.15±0.28^{a}	80.43±0.28^{a}
	30 °C	90.33±0.19^{c}	92.69±0.09^{d}	93.33±0.33^{d}	95.55±0.09^{d}
	35 °C	87.46±0.43^{d}	89.61±0.61^{c}	90.78±0.09^{c}	92.51±0.43^{c}
	40 °C	0.00±0.00^{a}	84.62±0.34^{b}	86.26±0.37^{b}	88.15±0.09^{b}
Fe^{2+} chelating (%)	Control (25 °C)	58.86±0.59^{b}	60.02±0.00^{a}	62.02±0.48^{a}	63.53±0.19^{a}
	30 °C	63.47±0.06^{d}	64.83±0.09^{c}	66.52±0.16^{c}	68.30±0.06^{d}
	35 °C	61.24±0.13^{c}	62.59±0.13^{b}	64.58±0.09^{b}	66.94±0.06^{c}
	40 °C	0.00±0.00^{a}	60.53±0.13^{a}	62.46±0.41^{a}	64.73±0.06^{b}

Data are presented as mean ± SD of three independent experiments. Different superscript letters within a column indicate significant differences ($P < 0.05$)

6.4.5 Kinetic studies of thermal elicitations of stress markers, phenylpropanoid triggering enzymes and accumulation of phenolic compounds

6.4.5.1 Accumulation kinetics of oxidative stress markers

The kinetic parameters for accumulation of oxidative stress markers are presented in Table 6.2. The accumulation rate k (h^{-1}) of H_2O_2 increased with elicitation temperatures, with treatment of 40°C eliciting the highest H_2O_2 rate constant of 0.95×10^{-2}. Thus, signifying 1.83-

fold increase in the rate of H_2O_2 production with increase in the elicitation temperatures (25 - 40 °C) applied to sprouts. Also, the determination coefficients (R^2) were very high at 0.99 (except 0.58 at 25 °C), implying that experimental data for H_2O_2 were well fit by the first order kinetic model under the tested temperatures.

Table 6.2: First order kinetic parameters of stress markers during thermal elicitation of common bean sprouts

Oxidative stress marker	Thermal elicitations (°C)			
	25	30	35	40
H_2O_2				
k [k × 10^{-2} (h^{-1})]	0.523±0.000	0.820±0.000	0.927±0.000	0.957±0.001
A_o	0.074±0.001	0.112±0.008	0.136±0.003	0.176±0.005
R^2	0.576	0.998	0.994	0.996
MDA				
k [k × 10^{-2} (h^{-1})]	0.417±0.001	0.510±0.000	0.550±0.001	0.785±0.001
A_o	10.689±1.403	38.913±2.273	44.535±2.886	58.087±3.986
R^2	0.979	0.921	0.922	0.971
Catalase				
k [k × 10^{-2} (h^{-1})]	0.350±0.000	0.883±0.002	0.517±0.001	0.290±0.000
A_o	6.370±0.004	42.909±1.335	35.296±0.586	6.127±1.122
R^2	0.960	0.978	0.994	0.998
Guaiacol peroxidase				
k [k × 10^{-2} (h^{-1})]	0.030±0.000	1.643±0.001	0.913±0.001	0.860±0.002
A_o	0.654±0.006	8.752±0.286	5.254±0.356	3.999±0.042
R^2	0.801	0.988	0.978	0.997

H_2O_2: hydrogen peroxide; MDA: malondialdehyde; k: rate constant, R^2: coefficient of determination

Similar to H_2O_2, increase in elicitation temperatures elevated the rate of MDA production. As shown in Table 6.2, the estimated rate constant, k (h^{-1}) for production of MDA at 25 °C was 0.417×10^{-2}. This rate increased significantly by 46.9% over a 15 °C temperature rise. Also, the determination coefficients (R^2) estimated for MDA production ranged from 0.92 to 0.98, suggesting that obtained experimental data well fitted the first order kinetic model under investigated temperatures.

Increasing thermal elicitation up to 30 °C resulted in an estimated rate constant k (h^{-1}) of 0.883×10^{-2} for catalase activation (Table 6.2). However, this rate was significantly reduced by 3-fold when temperature was increased by 10°C. Synchronizing with this trend was the result for guaiacol peroxidase activation. Elicitation temperatures up to 35°C estimated a rate constant k (h^{-1}) of 1.643×10^{-2} for its activation, with this observation decreasing by 47.67% when temperature increased up to 40 °C. Reduced activation rates observed for catalase and guaiacol peroxidase above 30°C can be attributed to elevated levels of H_2O_2 and MDA at higher temperatures.

6.4.5.2 Activation kinetics of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL)

Kinetic parameters for activation of PAL and TAL during thermal elicitation of common bean sprouts are shown in Table 6.3. Within investigated temperatures, the rate of PAL activation varied from 0.4900×10^{-2} to 1.40×10^{-2}. The first order rate constant k (h^{-1}) for PAL activation was statistically higher at the low temperature (30°C) compared to high temperature treatments (35 and 40 °C).

Table 6.3: First order kinetic parameters for activation of phenylpropanoid triggering enzymes under thermal elicitation of common bean sprouts

PPP enzyme	Thermal elicitation (°C)			
	25	30	35	40
PAL				
k [k × 10^{-2} (h^{-1})]	0.490±0.000	1.400±0.002	1.310±0.001	1.210±0.003
A_o	1.074±0.008	5.088±0.144	1.728±0.006	1.175±0.025
R^2	0.922	0.975	0.979	0.997
TAL				
k [k × 10^{-2} (h^{-1})]	1.140±0.001	2.190±0.008	1.480±0.002	0.180±0.001
A_o	0.531±0.030	3.571±0.414	1.829±0.149	0.099±0.042
R^2	0.701	0.995	0.961	0.943

PAL: phenylalanine ammonia-lyase; TAL: tyrosine ammonia-lyase; PPP: phenylpropanoid pathway; k: rate constant, R^2: coefficient of determination.

Corresponding to PAL activation, high elicitation temperatures presented reduced activation rate of TAL. The highest TAL activation rate of 2.190×10^{-2} was estimated at 30 °C, with this trend decreasing by about 91.8% when sprout elicitation temperature reached 40 °C. Overall, the estimated activation rates of PAL and TAL from this study suggest that elicitation temperatures >30°C induce lower expression of enzymes responsible for stimulating the phenylpropanoid pathway.

6.4.5.3 Accumulation kinetics of total phenolic acids, flavonoids and anthocyanins

As shown in Table 6.4, the rate of formation k (h^{-1}) of total phenolic acids, flavonoids and anthocyanins were dependent on elicitation temperatures. The highest rate formation for phenolic acids, flavonoids and anthocyanins were estimated as 6.2200×10^{-2}, 6.4400×10^{-2}

and 3.3800 × 10^{-2}, respectively. At temperatures >30°C, there were reductions in k (h^{-1}) formations for all evaluated phenolic compounds.

Table 6.4: First order kinetic parameters for formation of phenolic compounds under thermal elicitation of common bean

Phenolic compound	Thermal elicitation (°C)			
	25	30	35	40
TPA				
k [k × 10^{-2} (h^{-1})]	3.740±0.001	6.220±0.001	5.480±0.042	3.540±0.001
A_o	1.429±0.135	23.929±1.592	2.067±0.283	0.285±0.012
R^2	0.993	0.879	0.867	0.978
TFC				
k [k × 10^{-2} (h^{-1})]	0.870±0.001	6.440±0.002	1.480±0.004	1.050±0.003
A_o	1.387±0.067	92.877±0.413	78.521±6.849	16.420±0.401
R^2	0.815	0.949	0.998	0.706
ANTH				
k [k × 10^{-2} (h^{-1})]	0.050±0.001	3.380±0.008	0.730±0.002	0.460±0.003
A_o	8.011±0.052	13.120±0.518	9.096±0.359	0.812±0.006
R^2	0.808	0.965	0.891	0.730

TPA: total phenolic acids; TFC: total flavonoids contents; ANTH: total anthocyanins;
k: rate constant, R^2: coefficient of determination

These kinetic parameters are in synchrony with phenolic compounds accumulations discussed earlier, where 30°C elicited maximum concentration for all tested phenolic compounds. Also, the results are in synchrony with kinetic parameters of the phenylpropanoid triggering enzymes (PAL and TAL), where their estimated activation rates were optimum with 30 °C treatment. Thus, accounting for the observed maximum rate and percentage accumulations of phenolic compounds at sprout elicitation temperature of 30°C. Furthermore, the high

determination coefficients (R^2), which ranged from 0.88 to 0.99 (total phenolic acids), 0.71 to 0.99 (total flavonoids) and 0.73 to 0.96 (total anthocyanins), can be used to conclude that obtained experimental data were well fit by the first order kinetic model used to assess formation of phenolic compounds under the tested temperatures.

6.4.5.4 Activation energy for stress markers, phenolic triggering enzymes and phenolic accumulations during thermal elicitation of sprout

Temperature dependence for elicitation of phenolic compounds in common bean sprouts was estimated by the Arrhenius equation in Eq. 6.6. Among oxidative stress markers, the activation energies E_a (kJ/mol) for H_2O_2 and MDA were reported to be 15.7 and 41.6 kJ/mol, respectively, whereas E_a for antioxidant enzymes, i.e., catalase and guaiacol peroxidase, were estimated as 55 and 174 kJ/mol, respectively, (Table 6.5). This result explains why the rates of H_2O_2 and MDA formation were increasing with temperature rise, as well as why the estimated activation rate of catalase was higher compared to guaiacol peroxidase. It signifies that during the thermal elicitation of common bean sprouts, less energy was required for the formations of free radicals and lipid peroxidation compared to activation rates of antioxidant enzymes. Therefore, the demand to signal the biosynthesis of phenolic compounds as an alternative defense mechanism against accumulated stress markers (H_2O_2 and MDA).

In response to stress markers, the activation energies for activities of phenylpropanoid triggering enzymes were calculated as 149 and 221 kJ/mol for PAL and TAL, respectively. Low E_a (kJ/mol) observed for PAL explains its estimated high activation rate, compared to TAL. Thus, less energy was required by PAL to catalyse the deamination of phenylalanine into trans-cinnamic acids. Also, the activation energies calculated for total phenolic acids, flavonoids and

anthocyanins were 30.4, 64.0 and 209 kJ/mol, respectively, confirming the higher accumulation trend of total phenolic acids and flavonoids, compared to anthocyanins, as further confirmed in Table 6.4.

Table 6.5: Activation energy of stress markers, phenylpropanoid triggering enzymes and phenolic accumulations during thermal elicitation

Evaluated component	E_a (kJ/mol)	R^2
Stress marker		
H_2O_2	15.74±8.18	0.94
MDA	41.62±3.15	0.88
CAT	55.41±1.79	0.61
GPX	174.54±11.58	0.69
PPP triggering enzyme		
PAL	149.80±6.95	0.82
TAL	221.12±18.41	0.70
Phenolic compound		
TPA	30.40±1.76	0.84
TFC	63.98±1.76	0.93
ANTH	208.63±12.59	0.94

E_a: activation energy

6.5 Conclusion

From this study, thermal elicitation at 30 °C for 96 h significantly enhanced biosynthesis of phenolic compounds and their antioxidant capacities, by inducing oxidative stress at threshold levels that translated into maximum triggering of PAL and TAL for optimum stimulation of the phenylpropanoid pathway along the sprouting process. Moreover, elevated oxidative stress observed with 30°C elicitation was not negatively translated into the morphological characteristics of the developed sprouts, due to its significant inductions of CAT and GPX, compared to the control, 35 and 40 °C treatments. Also, from the kinetic modelling studies it was

confirmed that increasing sprouting time increased kinetic rates, k (h^{-1}), of all investigated biochemical and enzymatic mechanisms. Low E_a (kJ/mol) energies reported for PAL, total phenolic acids and flavonoids correlated with their maximum accumulation rates, k (h^{-1}), at 30 °C throughout the sprouting process, compared to TAL and total anthocyanins. Thus, thermal elicitation at 30°C can serve as a potential tool to enhance the composition of phenolic compounds and antioxidant capacities of common bean sprouts.

CHAPTER VII: General conclusions, contributions to knowledge and recommendations for future work

7.1 General conclusions

In the present study, chemical, ultrasonic and thermal elicitations combined with sprouting were used to enhance the biosynthesis of phenolic compounds and antioxidative properties of common bean (*Phaseolus vulgaris*). All investigated elicitors elevated oxidative stress markers (H_2O_2, catalase, guaiacol peroxidase and malondialdehyde), leading to higher expressions of phenylpropanoid triggering enzymes (phenylalanine ammonia-lyase and tyrosine ammonia-lyase) and maximum accumulation of total phenolic acids, flavonoids and anthocyanins in a sprouting time dependent manner.

Oxidative stress markers demonstrated varied responses to elicitors, with the highest content of H_2O_2 generated with 40 °C thermal treatment at 96 h of sprouting time, whereas overall catalytic activities of catalase and guaiacol peroxidase were best activated with 30 °C treatment at 96 h of sprouting. In response to oxidative stress, stimulation of the phenylpropanoid pathway also showed varied sensitivities depending on elicitor nature, with the highest catalytic activities of phenylalanine ammonia-lyase and tyrosine ammonia-lyase observed with 30 °C treatments, which was reflected in its highest accumulations of total phenolic acids, flavonoids and anthocyanins, compared to other investigated elicitors at the same sprouting time of 96 h. However, although 30 °C treatment resulted in highest accumulation of phenolic compounds, the overall greatest antioxidant capacities were demonstrated at 96 h sprouting time with 360 W (60 min) ultrasonic phenolic compound extracts. From kinetic modeling, sprout elicitation temperatures above 30 °C decreased accumulation of phenolic compounds and reduced

activation rates of antioxidant and phenylpropanoid triggering enzymes. Thus, this study showed that bioactivity of phenolic compounds is not only determined by their concentrations but also on the chemical structure of the individual phenolic compound elicited in the extract. Hence, further analyses on the profiling of phenolic compounds can better explain and correlate their structure to observed bioactivities.

Conclusively, ultrasonic treatment with 360 W (60 min) at sprouting time of 96 h was the best elicitation conditions to accumulate phenolic compounds with higher antioxidant capacities in common bean sprouts. Furthermore, morphological characterization showed that, controlled elicitation can also be a potential approach for the hydroponic, agricultural and nutraceutical food industries to produce natural ready-to-eat functional foods at a limited time.

7.2 Contributions to knowledge

1. This study clearly showed that, low temperature simple techniques such as ultrasonication and 30 °C sprouting can be cost-effective methods for the production of phenolic-rich foods with enhanced functional properties.
2. Bioactivity of phenolic compound extracts were found not only to be dependent on concentration but also on the individual types of phenolic compounds being elicited.
3. For the first time, this study demonstrated a link between stress accumulation, stimulation of phenylpropanoid triggering enzymes and accumulation of phenolic compounds along the sprouting phase of common beans.
4. To the food and agricultural industries, sprouting of seeds is time consuming and thus, expensive. It was demonstrated from this study that, ultrasonication is an efficient approach to enhance morphological properties of seeds at a reduced sprouting time.

5. Kinetic studies provided additional understanding on accumulation and reaction rates of stress markers and phenylpropanoid triggering enzymes towards the accumulation of phenolic compounds during thermal elicitation of common bean sprouts.

7.3 Recommendations for Future Work

- *Chemistry*

Combined elicitation techniques and profiling of elicited phenolic compounds

- *Bioavailability and bioactivity*

Impact of elicitation on *in-vitro* bioaccessibility of phenolic compounds. Predictive molecular docking to estimate *in-vitro* bioavailability and bioactivity of phenolic compounds

- *Molecular biology*

Genetic expressions of phenylpropanoid triggering enzymes and mRNA transcriptions during elicitations

- *Application*

Stability and bioactivity of elicited phenolic compounds through large scale hydroponic production and food formulations

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