

Original Research Article

Analysis of Seed Vigor Responses in Soybean to Invasive Silver Carp (*Hypophthalmichthys molitrix*) Protein Hydrolysate Treatments

ABSTRACT

Aim: To produce fish protein hydrolysates (FPH) from invasive silver carp (*Hypophthalmichthys molitrix*) under controlled hydrolysis conditions, and to examine the effects of FPH on seed vigor, using standard vigor tests.

Methodology: Soybeans were treated with FPH hydrolyzed for 1, 5.5 and 10 hrs with papain (FPH-Pa), pepsin (FPH-P) and trypsin (FPH-T), respectively. Overall vigor tests (accelerated aging and warm and cold germination dry weight, height, total phenolics and guaiacol peroxidase assessment-GuPx) were compared to a distilled-water control over a 12-day germination period.

Results: Seeds treated with FPH-P and FPH-Pa at 1 hr (23% degree of hydrolysis) elicited the greatest growth responses. FPH-Pa at 1 hr increased ($P=0.05$) weight (1.38 g) and height (53 mm) compared to water control (1.25g and 46.8mm, respectively). FPH-Pa at 1 hr also had the highest GuPx values, which are indicative of lignification. FPH-Pa appeared to stimulate lignification and thus enhance weight and height of the seedling. FPH-P elicited the greatest phenolic response, with the highest total phenolic content on day 4 (1.27 mg GAE/g FW) and day 12 (1.43 mg GAE/g FW) compared to water control (0.59 mg GAE/g FW on day 4, 1.10 mg GAE/g FW on day 8). Higher phenolic content may have protected against oxidation during accelerated aging vigor test, resulting in higher germination rates (53.8% germination) for soybeans primed with FPH-P at 1 h compared to water controls (32.2% germination). Most FPH treatments increased germination under warm conditions, compared to water control. GuPx values overall were higher in FPH-treated soybeans.

Conclusion: Results suggest that the use of FPH produced with the enzymes papain and pepsin at 1 hour of hydrolysis are comprised of free amino acids and peptides that are beneficial to the stimulation of the proline-linked pentose phosphate pathway, which enhanced the vigor parameters measured.

Keywords: Silver carp; fish protein hydrolysates; seed vigor; seed treatment

1. INTRODUCTION

Seed treatments have been used for centuries for a variety of purposes, including early germination and increased seedling growth and vigor. On-going research has led to development of treatments that improve seed vigor, which promotes overall heartier plants and increased crop yield [1]. Many types of seed primers are available, including inorganic salts such as potassium nitrate, and polyethylene glycol [1,2]. However, there is still a limited selection of seed primers certified for organic crop producers. Organic primers are essentially limited to hot water treatments and biocontrol agents which can be unstable over relatively short periods of storage and have had varying degrees of success in their ability to promote plant and seed vigor [3,4]. There is a significant need for organic certified agricultural products, as the demand for organic crops increases. Though the term organic encompasses many regulations and methods of sustainable production as set out by the United States Department of Agriculture (USDA), Organic food at its essence is defined as crops and foods that have been produced without use of synthetic inputs [5]. Organic food sales have increased since 2004 from an \$11 billion dollar industry to \$25 billion in 2011 [5]. The demand for organic food continues to grow; in 2012 alone, there was a 7.4% increase in organic food sales [5].

Fish protein hydrolysates (FPH) are classified as soluble by-products obtained from proteolytic hydrolysis of crude fish proteins [6]. Production of FPH has attracted attention due to their high nutritive value and improved functionalities arising from the production of short peptides and free amino acids during hydrolysis [7]. Commercial enzyme preparations are used to maximize FPH yield, with degradation products such as amino acids and peptide fractions varying based on the structural differences in the proteins and different enzymes used for hydrolysis [8-10]. Another important factor affecting the composition of FPH is the degree of hydrolysis (DH) that the native protein undergoes. The DH assists in determining the average peptide chain length and can be controlled by manipulation of the hydrolysis time, enzyme/substrate (E/S) ratio, the type of enzyme being used and the kind of the substrate (fish) [8,9].

Research on the use of FPH (lacking controlled hydrolysis) on crop growth is generally limited to its use as in-ground fertilizers. Mackerel hydrolysates have been demonstrated to increase the height and weight of pea plants by 9% and 15% respectively versus controls [11]. These increases were attributed to an increase in production of phenolic compounds when seeds were treated with FPH. Phenolic compounds are known to improve the strength of plant cell walls during the lignin polymerization process of growth [12,13]. The increase in production of phenolic compounds as a result of FPH treatment is also thought to enhance root development and increase the height and weight of soybean, tomato and corn seedlings [14]. It is important to note, however, that in these studies, the FPH preparation conditions such as enzyme used, enzyme/substrate ratio (E/S), degree of hydrolysis (%DH), and hydrolysis time) were not specified.

Silver carp (*Hypophthalmichthys molitrix*), an underutilized and invasive fish found in the waterways of 18 different American states, including the Mississippi River System where there is potential for loss of biodiversity resulting from the silver carp colonization [15]. Silver carp species are now considered "injurious wildlife" by the United States Fish and Wildlife Service [16]. New recommendations by the Asian Carp Regional Control Committee (ACRCC) for control of silver carp populations primarily relate to the harvest and use of these fish rather than piscicidal options such as chemical and electrochemical barriers [17].

The objective of this study was to produce FPH from invasive silver carp under controlled hydrolysis conditions (DH, E/S ratio, enzyme used), and to examine the effects of the FPH on soybean seed vigor, using standard vigor tests (accelerated aging, warm germination and cold germination, weight, height, total phenolics and guaiacol peroxidase assessment). The ultimate goal was to be able to use these fish to develop an economic agricultural product to improve seed vigor for organic crop producers while aiding in the control of an invasive fish species.

2. MATERIAL AND METHODS

2.1. FPH preparation

Fresh silver carp (*Hypophthalmichthys molitrix*) were harvested from the Wabash River (Lafayette, IN, USA). The fish were transported on ice within 24 h to the Purdue University Food Science Department where they were beheaded, eviscerated, filleted and immediately frozen at -20 °C for later use. FPH were produced as described by Liceaga-Gesualdo and Li-Chan (1999) with some modifications. The silver carp fillets were thawed, and mixed with water (1:2 w/v fish-distilled water), then homogenized in a commercial blender (Waring Heavy Duty Blender 4L, Sigma-Aldrich, St. Louis, MO, USA) at medium speed (18000 rpm) for 3 min. The enzymes pepsin (E.C.3.4.23.1), papain (E.C. 3.4.22.2) and trypsin (E.C. 3.4.21.4) (Sigma-Aldrich, St. Louis, MO, USA) were added separately to the slurries at 2.5% (w/w) of the protein content in the fish. Each mixture was agitated in a water bath maintained at 55°C and underwent hydrolysis for 1.0, 5.5 and 10 hours. After hydrolysis, samples were pasteurized (85°C for 15 min) to inactivate the enzyme and

centrifuged (Avanti J-26S Centrifuge, Beckman-Coulter INC. CA, USA) at 15000g for 15 min and 4°C to separate the insoluble fraction. The FPH supernatant was collected from each enzyme treatment [pepsin (FPH-P), trypsin (FPH-T) and papain (FPH-Pa)] and stored at -80°C until use. A FPH control (FPH-C) was prepared using the same process conditions as above except that the fish slurry was immediately pasteurized without enzyme addition.

The best performing FPH elicitors as determined from preliminary seed vigor trials (data not shown) were chosen for subsequent trials.

2.2. Degree of hydrolysis (DH)

The DH was calculated using the trinitrobenzene sulfonic acid (TNBS) method described by Adler-Nissen (1986) with modifications by Liceaga and Li-Chan (1999). Briefly, 10 mL aliquots from each trial were mixed with 10 mL of 24% trichloroacetic acid (TCA) and centrifuged for 5 min at 12100g. Aliquots (0.2 mL) from the supernatant were mixed with 2mL of sodium borate buffer (0.2 M at pH 9.2) and 1.0 mL TNBS (4.0 mM) and then incubated for 30 min in the dark at room temperature. This was followed by the addition of 1 mL of 2.0 M NaH₂PO₄ containing 18 mM Na₂SO₃. The absorbance was read at 420 nm using a UV-Vis spectrophotometer (Beckman-Coulter DU640 UV-Vis Spectrophotometer, Beckman-Coulter Inc. CA, USA). DH was calculated using the equation:

$$\%DH = h/h_{tot} \times 100$$

where h represents the number of peptide bonds broken and h_{tot} represents the total number of peptide bonds present. In fish protein concentrate, h_{tot} was assumed to be 8.6 meq/g [8].

2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on FPH-P, FPH-Pa and FPH-T. To 40µL of each FPH sample, 2 µL 2-mercaptoethanol, 20 µL 10% SDS, and 5 µL tracking dye (0.05% bromophenol blue) in 10 mM Tris/HCl, 1 mM EDTA, pH 8.0 was added. Samples were heated in a boiling water bath for 10 min before being centrifuged at 10000g for 5 min. Electrophoresis was performed using a PhastSystem™ (G.E. Healthcare Biosciences, Pittsburgh, PA, USA) with 10-15% gradient polyacrylamide PhastGels. After electrophoresis, the gels were stained for 45 min with Coomassie blue (0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid) and destained with 30% methanol and 10% acetic acid until bands were visible. To preserve the gels, gels were placed in a solution of 10% acetic acid and 5% glycerol. The approximate molecular weights were determined using high range molecular weight standards (Sigma-Aldrich, St. Louis, MO, USA).

2.4 Free amino acid composition

Free amino acid composition analysis of FPH samples was completed by Bindley Discovery Park Proteomics Facility at Purdue University using a Beckman HPLC (126 pump, 166 detector, 507 autosampler) with a Waters AccQ Tag amino acid analysis column and UV detector, calibrated with amino acid standards (Pierce, Rockford, IL).

2.5 Seed treatment

Seed treatments and preparation of plant materials were in accordance with methods modified from those described by Randhir and Shetty (2005). Seeds of the soybean (*Glycine max*) variety Viking 2265 (Johnny's Seeds, Waterville, ME) were used for this study. Seeds were washed using a 1:5 ratio of bleach (5.25% v/v sodium hypochlorite) to distilled water to remove possible contaminants and then rinsed twice for 1 min each with distilled water to remove excess bleach. Decontaminated seeds were placed into 100 mL of treatment solution. Each treatment solution contained 5 mL/L (v/v) FPH supernatant to distilled water, containing 107.6 ug/mL of protein per FPH treatment solution. The primed seeds were incubated for 24 hours in an orbital shaker set at 120 rpm and excess water was drained. This method of seed treatment and priming was used for all vigor assessment trials.

2.6 In-Vitro Soybean Seed Germination

A total of 50 seeds per FPH treatment were placed in individual treatment solutions of 5 mL/L (v/v) FPH supernatant to distilled water for 24 hours. Treated seeds were then placed in Petri dishes lined with filter paper and covered with aluminum foil to block light during germination. Seeds were incubated at room temperature (~22°C) and sprayed four times daily with sterile distilled water to maintain high moisture. Seeds primed only with distilled water were used as controls. Germination data was collected on day 4, 8 and 12. There were three replicate trials per treatment for a total of 150 seeds per treatment.

2.7 Growth Chamber Seed Germination

A total of 50 soybean seeds per FPH treatment were placed in sterile potting soil, approximately (2.5 cm) deep and allowed to germinate in a growth chamber over a 12-day period. Seedlings were evaluated on day 4, 8 and day 12 for fresh weight (including roots), length of seedling (from root to shoot) and root to shoot length ratio. All tests were completed in duplicate. Temperatures in the growth chamber were maintained at 30°C during the day and 20°C at night. Relative humidity was maintained at 70% RH.

2.8 Warm seed germination

Percent germination of FPH-treated seeds was conducted using the ragdoll method, as outlined by the United States Department of Agriculture [18]. Treated soybeans (triplicate trials with 50 seeds per treatment and trial) were spread out along the center of two sterile paper towels. The paper towels were moistened with sterile water and then tightly rolled into tubes. The tubes were placed in plastic bags and incubated for 4 days at 30°C. Germination was defined as emergence of the radical. Percent germination was calculated as the number of seeds germinated versus the total number of seeds.

2.9 Cold Seed Germination

A modified 'shoebox method' for cold germination as used by the Indiana Crop Improvement Association (ICIA) and the Oregon State University Seed Laboratory and in accordance with methods developed by the Association of Official Seed Analysts (AOSA) was used to test seed vigor under cold stress conditions (Association of Official Seed Analysts, 1983; Indiana Crop Improvement Association, 2014; Oregon State University, 2014). Soybean seeds were treated with FPH solutions as was used for in-vitro seedling testing. After draining excess water, seeds were placed on sterile paper towels and dried at 30°C for 16 hours. Seeds were then placed in 2.5 cm of sterile vermiculite moistened to a 75% saturation level and stored at 10°C for 7 days. On day 8, the temperature in the growth chamber was increased to 25°C, and maintained for an additional 5 days. Percent seed germination was determined on day 12.

2.10 Accelerated aging test

The accelerated aging test described by the Iowa State University Seed Testing Laboratory in accordance with methods established by the International Seed Testing Association (ISTA) and AOSA was used (Association of Official Seed Analysts, 1983; Indiana Crop Improvement Association, 2014; Iowa State University, 2014). Both control and FPH-treated seeds were placed on sterile paper towels and dried at 30°C for 16 hours. Seeds were then placed in trays elevated above 40 mL of water in a sealed box. The boxes were then placed in a growth chamber and exposed to high temperature (45°C) and high moisture (approx. 100% relative humidity) for 72 hours. The seeds were then subjected to the warm germination protocol previously described. Percent germination was determined after 4 days.

2.11 Total phenolics assay

Total phenolic content was quantified using the Folin-Ciocalteu reagent as outlined by Andarwulan and Shetty (1999). Sprout and leaf tissue from the seedlings were extracted using 99.5% ethanol (1ml ethanol/50 mg fresh weight) and distilled water. The tissue was homogenized using a mortar and pestle and centrifuged for 10 min at 17000g. One mL of supernatant was added to 0.5 mL of Folin-Ciocalteu reagent, 1mL of 99.5% ethanol and 5 mL distilled water and incubated for 5 min at room temperature (approximately 22°C). After incubation, 1 mL of 5% Na₂CO₃ (w/v) was added to each sample. The resulting solution was vortexed and incubated in the dark at room temperature (~22°C) for 60 min. Absorbance was read at 725 nm using a UV-Vis spectrophotometer. Total phenolics was calculated as gallic acid equivalent (GAE) using the equation:

$$T = C \times (V/M) [2]$$

where T represents the total phenolic content in mg/g, C is the concentration of gallic acid as established by the gallic acid calibration curve in mg/mL, V is the volume of leaf extract used in mL and M is weight of the leaf extract in g.

2.12 Preparation of enzyme and protein extracts

Protein was extracted based on modifications to the protocol by Horii et al. (2007b). Tissue (100 mg) derived from the shoots of soybean was placed in 2 mL of the enzyme extraction buffer containing 0.5% (w/v) polyvinylpyrrolidone, 3mM EDTA and 0.1 M potassium phosphate buffer and ground using a chilled mortar and pestle. The tissue-buffer mix was centrifuged for 10 min at 6000g and held at 4°C. The supernatant was then used for the protein and guaiacol peroxidase (GuPX) assays.

2.13 Guaiacol peroxidase (GuPX) assay

GuPX was determined as described by McCue et al. (2000). Supernatant (50 uL) from the extraction buffer / tissue homogenate solution was placed into a GuPX assay solution containing 0.1 M potassium phosphate buffer (pH 6.8), 20 mM guaiacol solution and 0.2 mM hydrogen peroxide. Total volume was 1 mL. The increase in absorbance at 470 nm was recorded over a period of 5 minutes and indicated the production of tetraguaicol. The GuPX solution without the tissue homogenate was used as a blank. GuPX activity was reported as nmol tetraguaicol/min/mg protein.

2.14 Protein Determination

Protein content of soybean tissue was determined by the bicinchoninic acid protein assay (BCA) according to manufacturer's protocol using bovine serum albumin as a standard (Thermo Scientific Pierce Pierce Biotechnology, Rockford IL). Protein content was used for the calculation of GuPX activity.

2.15 Statistical analysis

All data were subjected to an analysis of variance (ANOVA) using Minitab® 16 software (Minitab®, PA, USA) to determine whether the treatments had a significant effect on seed germination and vigor. The difference between treatments was analyzed by Fisher's test with statistical significance at $\alpha = 0.05$ unless otherwise noted.

3. RESULTS AND DISCUSSION

Our study sought to further demonstrate the ability of FPHs to elicit phenolic responses in seeds during conditions of stress and determine which treatments used to produce the FPHs were most beneficial [19].

After 1 hour of hydrolysis, pepsin was able to hydrolyze the crude fish protein to a DH of 23.3% (Table 1) and increasing the hydrolysis time to 5.5 hours had no additional increase on the DH (23.5%). Trypsin hydrolyzed the proteins to a higher DH (26.4%) than pepsin after 5.5 hours with a slight increase in DH after 10 hours (27.5%). Papain hydrolyzed the fish protein more extensively than either pepsin or trypsin, yielding 23.8% DH after 1 hour and 36.3% DH after 10 hours. These results are consistent with findings by Ng and Khan (2012) who reported that pepsin and trypsin had similar DH when hydrolyzing palm kernel expeller proteins. By using different enzymes and varying hydrolysis times, we produced hydrolysates containing different peptide fractions. These differences contributed to the variance observed in seedling vigor responses. The differences in substrate preferences of the enzymes used in protein hydrolysis resulted in distinct hydrolysates that stimulated plant growth unique to the individual hydrolysates. As illustrated by the SDS-PAGE profiles, FPH-P hydrolysed for 1 hour, showed 2 clear bands in the 200 and 66 kDa range (Fig. 1, lane 2). As hydrolysis continued, these bands dissipated into the lower molecular weight range (lanes 3 and 4). In contrast, FPH-Pa yielded only low molecular weight peptides (lanes 5, 6, 7) even at 1 hour of hydrolysis, which is consistent with the biochemical nature of papain, which hydrolyzes a broader spectrum of protein bonds compared to both trypsin and pepsin [10]. In the case of FPH-T, separation of peptides was similar for all hydrolysis times; banding occurred at 66 kDa, 200 kDa and 116 kDa for all three trypsin hydrolysis times (data not shown). Dense banding at lower molecular weight markers occurred in FPH-T hydrolyzed for 10 hours confirming the presence of smaller peptides in proteins hydrolyzed for longer periods of time.

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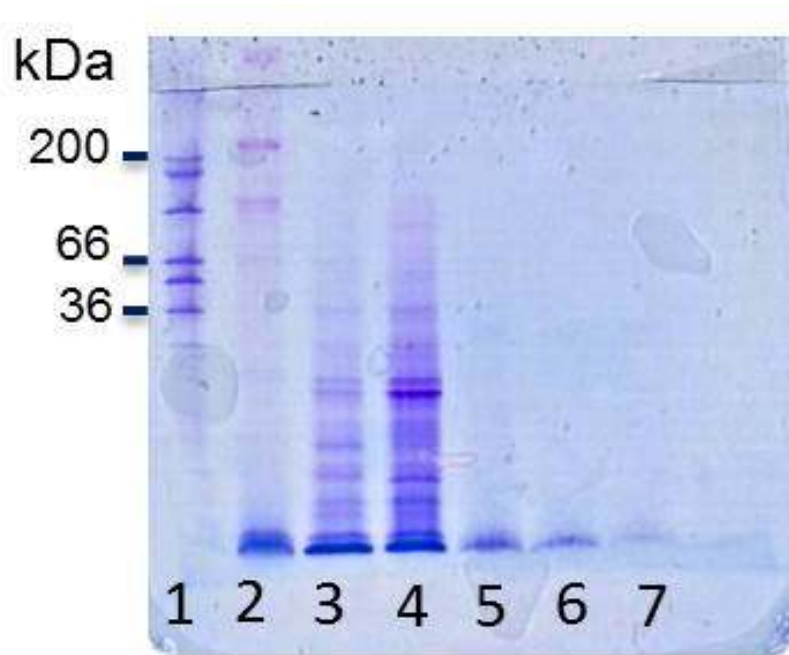


Fig. 1: SDS-PAGE profiles of FPH-P (lanes 2, 3, 4) and FPH-Pa (lanes 5, 6, 7) for 1, 5 and 10 hours, respectively. Wide range molecular weight protein standards (lane 1) representing 36 to 200 kDa. Treatment legend is: FPH-P = pepsin hydrolyzed proteins, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed proteins, Water control = seeds soaked in distilled water.

Table 1. Average percent (%) germination^a of seedlings by treatment and vigor tests.

Treatment ^b	Hydrolysis Time (hours)	DH (%)	Warm Germination	Cold Germination	Accelerated Aging
FPH-P	1	23.3	91.0 (4.4)A	47 (43.8)	53.8 (15.9)A
	5.5	23.5	80.8 (11.3)AB	49 (21.2)	28.9 (0.48)BC
FPH-T	5.5	26.4	88.8 (4.5)A	73 (7.1)	23.9 (2.67)BC
	10	27.5	89.9 (4.0)A	64 (8.5)	35.5 (0.76)BC
FPH-Pa	1	23.8	85.8 (1.8)A	64 (19.8)	19.9 (3.57)C
	10	36.3	85.7 (9.3)A	67 (4.2)	37.8 (10.0)AB
Water Control			72.7 (2.3)B	61 (18.4)	32.2 (2.59)BC

^a Values in parentheses represent the standard deviation (n=3) associated with each data set. Data bearing different uppercase letters (A, B, C) indicate significantly different ($P = .05$).

^b FPH-P = pepsin hydrolyzed proteins, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed proteins, Water control = seeds soaked in distilled water.

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In order for plants to synthesize proteins necessary for growth, essential amino acids must be readily available [20]. During hydrolysis, the original protein source is fractionated into smaller peptides and free amino acids [8], which increases the amount of ammonia and soluble nitrogen compared to the original native protein [21-23]. Nitrogen is used extensively as a plant fertilizer and the increased availability of nitrogen from the FPH to the plant cell for synthesis of essential amino acids likely would lead to increased seedling vigor.

Previous studies have shown that foliar applications of amino acids increases plant growth parameters such as number of leaves, fruits, height of plant and root structure [12,14,19,20,24]. The amino acid proline specifically has been shown to alleviate abiotic stressors during plant growth [13]. Hydrophilic amino acids such as lysine (LYS) and asparagine (ASP) (Table 2) are more easily absorbed into plant cells than peptides due to their smaller size and the differences in polarity and hydrophobicity of peptide fractions [25]. These amino acids also increase the concentration of osmo-regulatory components in plant cells [24]. In our study the increased availability of free amino acids as a result of hydrolysis elicited a greater growth response as compared to water controls.

Table 2. Free Amino acid composition (g/100 g) by treatment^a

	FPH-T		FPH-P		FPH-Pa	
Amino Acid	5.5 ^b	10	1	5.5	1	10
PHE	6.02	3.04	2.12	1.78	3.64	3.95
ILE/LEU	6.69	5.71	4.00	3.14	6.62	5.36
TRP	1.81	1.19	0.77	0.65	1.37	1.82
MET	13.9	9.90	7.90	5.58	10.7	19.0
VAL	25.0	28.2	18.2	18.0	16.6	22.2
PRO	1.78	1.60	7.08	8.61	5.55	3.91
TYR	3.23	2.38	1.36	1.31	2.02	3.62
THR	1.81	2.49	4.01	4.08	3.51	2.50
ALA	3.22	5.16	7.20	6.17	6.56	5.30
ARG	3.94	0.11	0.98	1.03	0.32	3.50
HIS	1.91	1.88	2.77	5.11	4.59	1.42
GLN	12.9	15.0	3.92	3.21	5.20	6.62
GLY	4.60	8.49	25.2	33.3	24.4	8.90
ASN	0.00	0.00	0.00	0.00	0.00	0.00
SER	0.73	0.71	2.21	0.39	1.38	0.99

LYS	8.00	10.4	4.89	4.12	4.83	4.27
CYS-CYS ^c	0.08	0.04	0.04	0.03	0.03	0.06
GLU	2.61	2.83	4.55	3.03	1.22	3.28
ASP	1.75	0.82	2.74	0.47	1.44	3.37

^aFPH-P = pepsin hydrolyzed proteins, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed proteins.

^bHours of hydrolysis (1, 5, or 10 hrs).

^cCysteine is underestimated since pre-derivatization was not performed.

Elicitors such as FPH have been documented to stimulate plant metabolic pathways when applied to seeds as priming agents [13,26]. Previous research has shown that the proline-linked pentose phosphate pathway (PPP) is stimulated predominantly by the high proline and glutamic acid content of the FPH, and therefore likely responsible for the increase in plant phenolic content [27]. Previous studies also show that when plants are placed under stress situations, as we have done by germinating our seedlings for longer periods of time in the dark with the addition of an elicitor, synthesis of phenolic compounds increases ([27]. Phenolics may serve to protect plants in situations in which they have been stressed by producing antioxidants with reactive oxygen species (ROS) scavenging capabilities (Randhir & Shetty, 2005). Simple phenolic compounds, as measured by GuPX activity, are also important in the lignification of cell walls [28].

On day 4, seeds treated with FPH-P at 1 hour of hydrolysis produced higher totals of phenolic compounds (1.27 mg GAE/g FW) than the water control (0.59 mg GAE/g FW) (Fig. 2). The necessity for cellular protection via increased lignification of cell walls is increased in stressful environments, which the seedlings were exposed to during dark germination, such as in the accelerated aging and cold germination trials. This is reflected by the significantly higher ($P = .05$) percent germination in the accelerated aging test for seeds treated with FPH-P at 1 hour of hydrolysis (53.8 %) as compared to 32.2% for water controls which correlated with the higher phenolic content of FPH-P at 1 hour of hydrolysis at day 4 (Fig. 2). Accelerated aging of soybeans and other oil seeds has been found to increase lipid peroxidation and accelerate damage due to free radicals [29]. The increased total phenolic content, but lower GuPX content (Fig. 3) of the treated seeds suggests that the pentose-phosphate pathway was stimulated and phenolic compounds present in the seed were diverted principally to increasing ROS antioxidant and defense activities of the cells on day 4. This in turn may enhance vigor during stress caused by heat and excessive humidity as in the situation of our accelerated aging test. Stimulation of the pentose-phosphate pathway was also likely increased in the seedlings during the warm germination trials (Table 1), as percent germination was significantly higher ($P = .05$) for both FPH-P at 1 hour (91.0%) and FPH-Pa at 1 hour (85.8%) compared to water controls (72.7%). Seedlings treated with both of these FPH treatments also had the highest phenolic content on day 4 (Fig. 2).

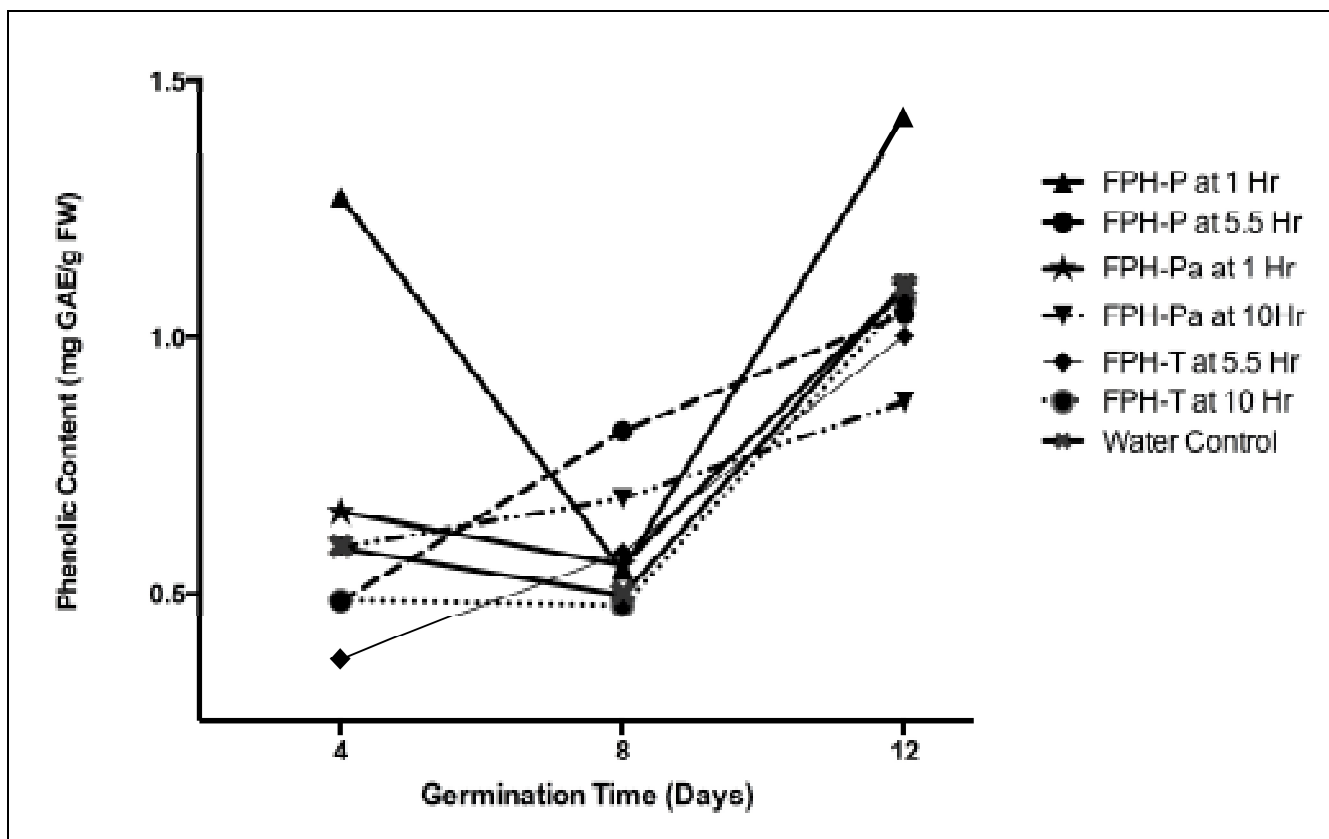
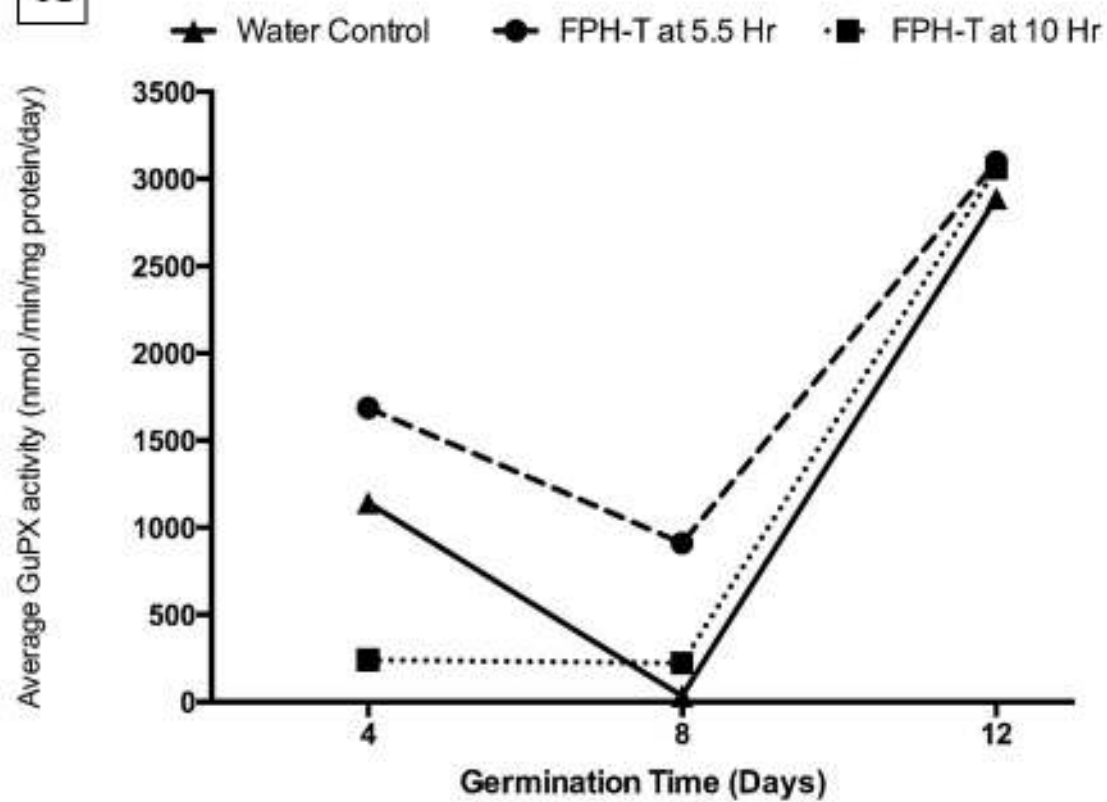


Fig. 2. Effect of FPH treatments on total phenolic content in mg Gallic Acid Equivalents (GAE) of soybean seedlings as compared to water primed seedlings: (a) FPH-P (b) FPH-T (c) FPH-Pa. Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled water.

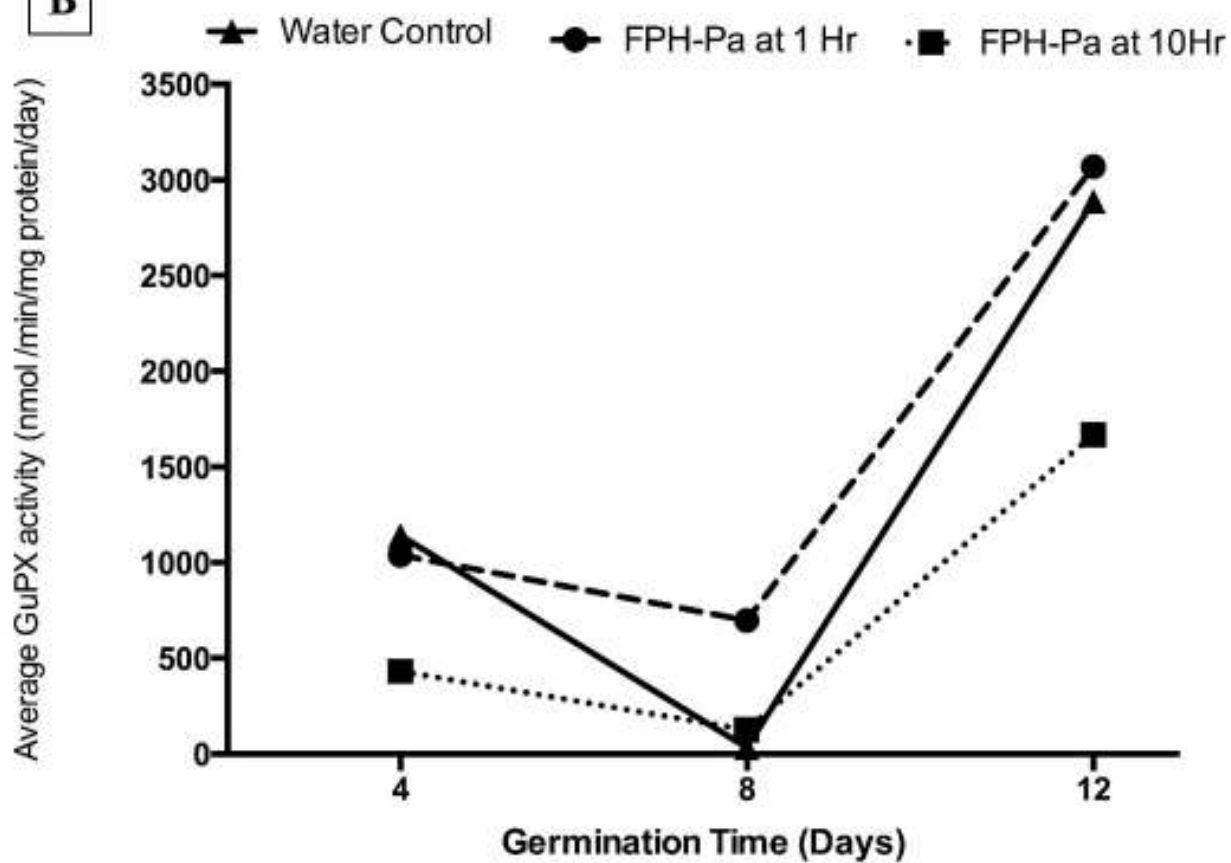
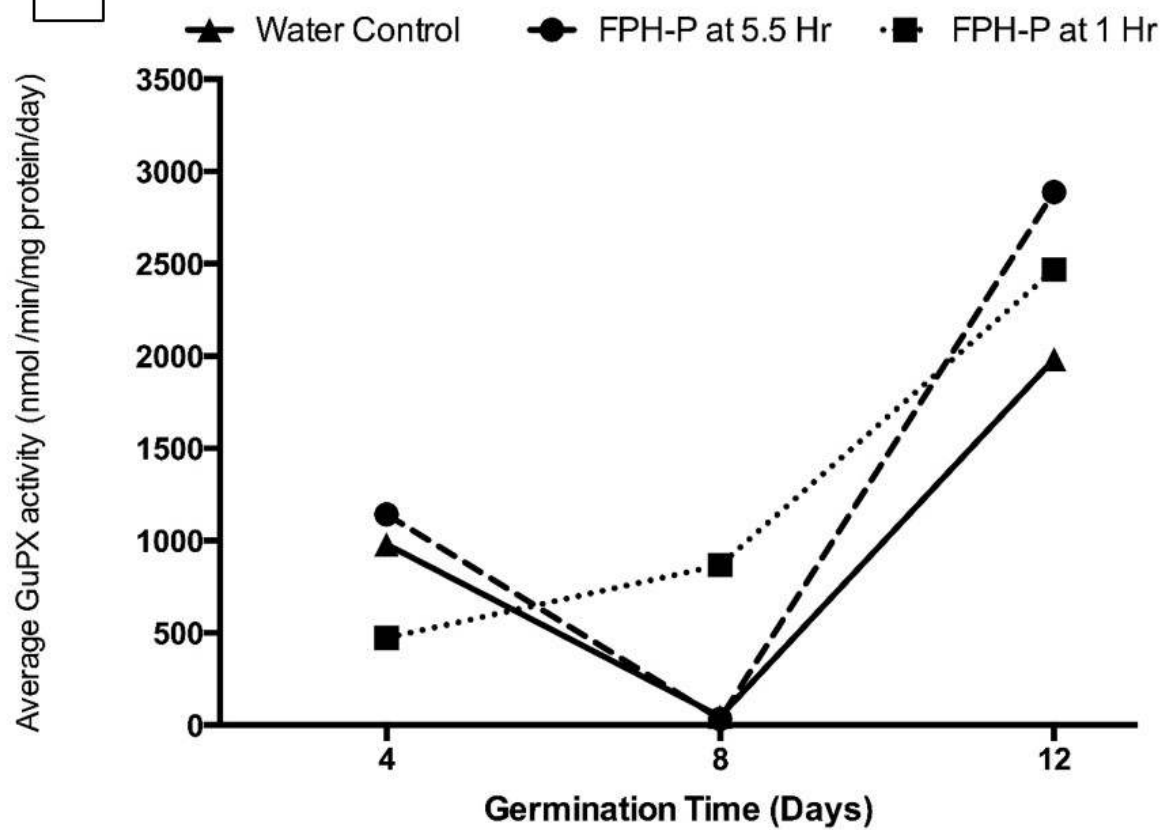
Total phenolic content decreased on day 8 in seeds treated with FPH-P at 1 hour, FPH-Pa at 1 hour and FPH-T at 10 hours. Other FPH treatments and the water control had consistent increases in phenolic content. GuPx concentration, however, decreased on day 8 for all treatments (Fig. 3 A-B) with the exception of FPH-P at 1 hour of hydrolysis (Fig. 3 C), also suggesting that the cells might be using the phenolic compounds as antioxidants. Despite decreases in GuPX content, FPH-Pa at 1 hour showed longer seedlings at both day 8 (30 mm) and day 12 (53 mm) germination times as seen in Fig. 4 compared to the 12-day water control (28 mm total length on day 8, 46.8 mm total length on day 12). Additionally, FPH-Pa at 1 hour produced heavier seedlings (Fig. 5) on day 12 (1.38 g/seedling) compared to water controls (1.25g/seedling on day 12). Both weight and length values reflect a larger GuPX value for FPH-Pa at 1 hour of hydrolysis on both day 8 and day 12 compared to water control. Overall, soybeans treated with FPH consistently had higher GuPX values on day 8 and day 12. Seeds treated with FPH-P at 1 hour of hydrolysis had increased GuPX content over the entire germination period, which is consistent with an increase in the pentose phosphate pathway and the generation of ROS antioxidant defenses to peroxidation in the initial stages of germination. However, exhaustion of these phenolics by day 8 might lead to a more extensive use of the compounds for growth and lignification [30].

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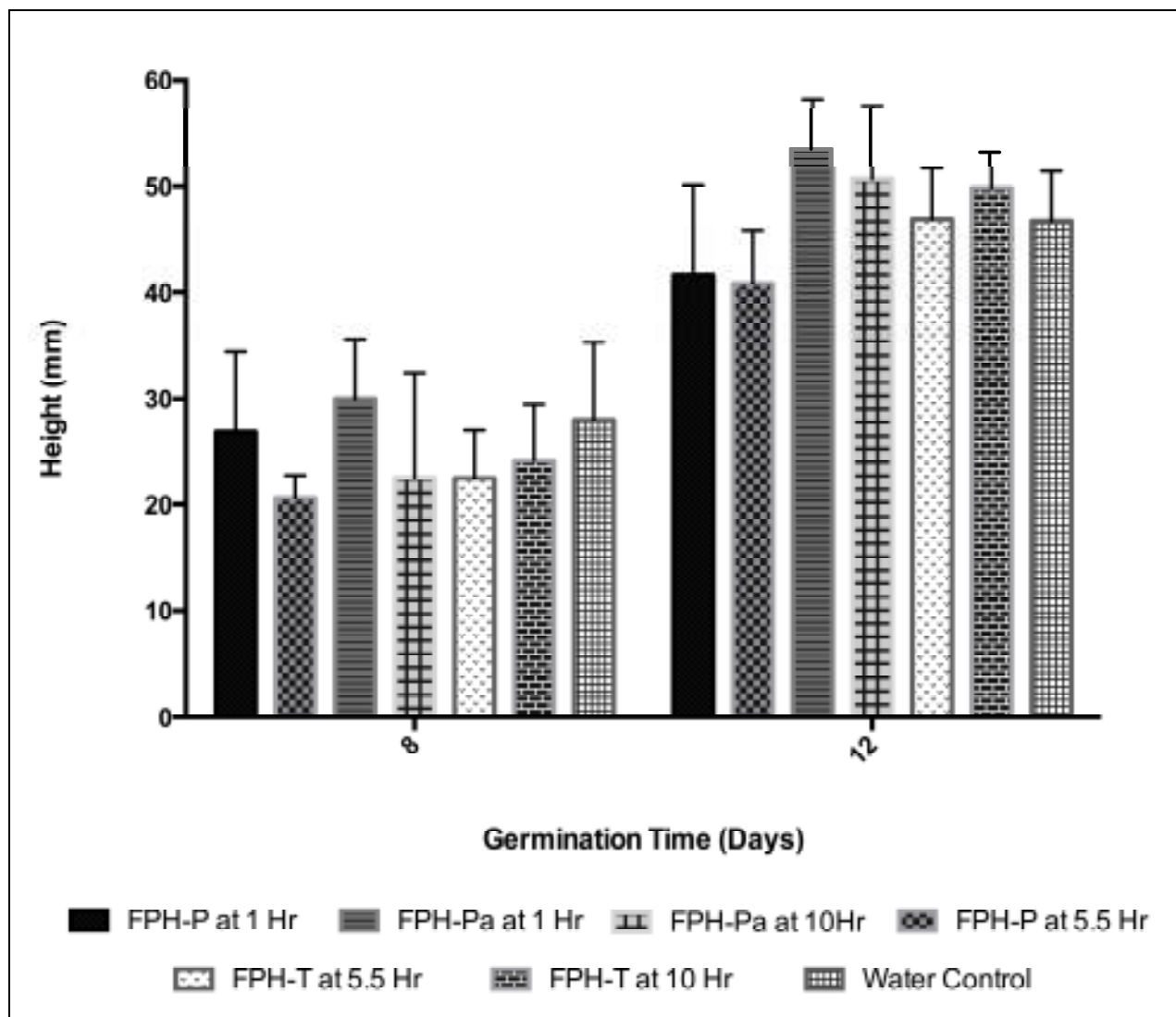
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308 **Fig. 3:** Effect of FPH treatments on Guaiacol Peroxidase (GuPX) Activity in nmol tetraguaiacol per minute per mg of
309 protein of FPH-T treated soybean seedlings as compared to water primed seedlings: **(A)** FPH-T = trypsin hydrolyzed; **(B)**
310 FPH-Pa = papain hydrolyzed; **(C)** FPH-P = pepsin hydrolyzed, at different time intervals; Water control = seeds soaked in
311 distilled water.

312 After 12 days of germination, both total phenolics and GuPX content increased for all treatments (Fig. 2 and 3), which
313 could reflect increased cellular synthesis of phenolic compounds and metabolic efforts being diverted to lignification and
314 plant growth [12,19,30]. No differences were found between phenolic contents for treatments on day 8 or day 12;
315 however, FPH-P at 1 hour did have a higher phenolic content on day 12 (1.43 mg GAE/g FW compared to 1.10 mg
316 GAE/g FW for the water control).

317 When subjected to cold stress, no significant differences were found in final germination percentages between soybean
318 seeds treated with FPH or water control (Table 1). FPH-T at 5.5 hours of hydrolysis (73.0% germination) appeared to
319 increase the percent germination compared to other treatments and the water control (61.0% germination), however it
320 was not statistically different. Cold stress decreased the overall germination for all treatments. Cold stress elicits different
321 metabolic responses from those caused by heat stress and dark stress [31]. Plants affected by cold stress use calcium-
322 gated channels to mediate the expression of the hormone abscisic acid (ABA). ABA further regulates genes involved with
323 physiological adaptations to the cold, such as delayed embryogenesis and closure of stomata to minimize fluid losses
324 [29]. The proline-linked pentose phosphate pathway (PPP) does not come into effect during periods of cold or drought
325 stress [31], therefore the FPHs do not have the same effect on the metabolic pathways regulating cold stress.

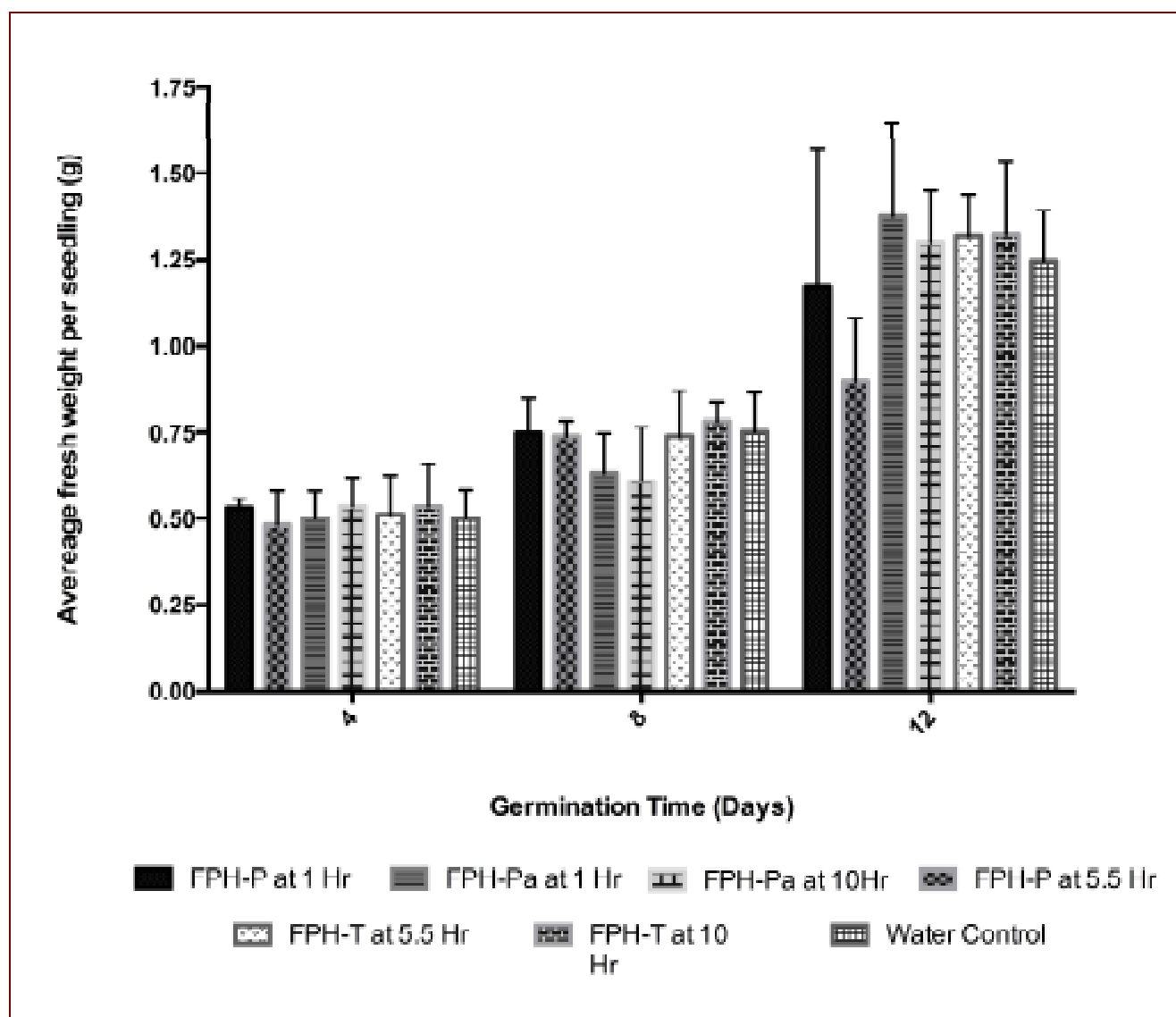


326 **Fig. 4.** Effect of FPH treatments and FPH-C on soybean seedling lengths as compared to water control seedlings. Bars
327 represent average values $\pm$ SD: **(a)** FPH-T **(b)** FPH-P **(c)** FPH-Pa. Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-
328

329 T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled
 330 water.

331

332 FPH-P at 1 hour of hydrolysis appears to be the most effective at stimulating the PPP. Pepsin has broad specificity,
 333 preferentially cleaving aromatic or carboxylic L amino acids, or at the C terminal of phenylalanine, leucine or glutamic acid
 334 [10]. FPH-P at 1 hour of hydrolysis contained free proline (7.08 g/100g protein), glutamine (3.92 g/100g protein protein)
 335 and glutamic acid (4.55 g/100g protein) (Table 2). Glutamic acid and glutamine have been shown to improve seedling
 336 vigor and elicit responses from the PPP and therefore could be responsible for the improvement of seedling vigor in this
 337 study [19]. FPH-Pa at 1 hour of hydrolysis was also able to improve some aspects of seedling vigor. Papain has a large
 338 active site and a lack of defined specificity [10]. Amino acids such as proline, glutamine and glutamic acid were be
 339 released during hydrolysis with papain, aiding in elicitation of the PPP and improving overall vigor; FPH-Pa at 1 hour of
 340 hydrolysis contained 5.55 g, 5.20 g and 1.22 g/100g protein of free proline, glutamine and glutamic acid, respectively. On
 341 the other hand, seedling vigor was improved only minimally when soybean seeds were treated with FPH-T. Trypsin is
 342 substrate specific, and does not cleave near the amino acid proline [10]. This is a detriment to the efficacy of FPH-T to
 343 stimulate the PPP, as proline is integral in the function of this metabolic pathway ([26]. FPH-T treatments contained less
 344 free proline, than other treatments, but contained more free glutamine (Table 2).



345

346 **Fig. 5.** Effect of FPH treatments and FPH-C on soybean seedling weights as compared to water control seedlings. Bars
 347 represent average values $\pm$ SD: (a) FPH-T (b) FPH-P (c) FPH-Pa. Treatment legend is: FPH-P = pepsin hydrolyzed, FPH-
 348 T = trypsin hydrolyzed, FPH-Pa = papain hydrolyzed, at different time intervals; Water control = seeds soaked in distilled
 349 water.

4. CONCLUSION

Protein hydrolysates derived from silver carp stimulated growth of soybean seedlings when applied as a seed primer. FPH created using both pepsin (23.3% DH) and papain (23.4% DH) led to shorter peptides and released free proline, glutamine and glutamic acid. This resulted in stimulation of the PPP, which increased phenolics responsible for protection against peroxidation. FPH-P and FPH-Pa, hydrolyzed for shorter periods of time (ie. 1 hour) at an E/S ratio of 2.5% have an ideal free amino acid and peptide composition for the overall stimulation of seedling vigor. With controlled-hydrolysis FPH have potential to be used as organic seed treatments and as an economically feasible value-added product that aids in controlling an invasive fish species. Further studies will be required to investigate the potential antioxidant activity of the peptides generated from the FPH, as well as their stability for applications in seed vigor enhancement.

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