Improving phenolic bioactive-linked anti-hyperglycemic functions of dark germinated barley sprouts (*Hordeum vulgare* L.) using seed elicitation strategy

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Abstract Sprouts of cereal grains, such as barley (*Hordeum vulgare* L.), are a good source of beneficial phenolic bioactives. Such health relevant phenolic bioactives of cereal sprouts can be targeted to manage chronic hyperglycemia and oxidative stress commonly associated with type 2 diabetes (T2D). Therefore improving phenolic bioactives by stimulating plant endogenous defense responses such as protective pentose phosphate pathway (PPP) during sprouting has significant merit. Based on this metabolic rationale, this study aimed to enhance phenolic bioactives and associated antioxidant and anti-hyperglycemic functions in dark germinated barley sprouts using exogenous elicitor treatments. Dark-germinated sprouts of two malting barley cultivars (Pinnacle and Celebration), treated with chitosan oligosaccharide (COS) and marine protein hydrolysate (GP), were evaluated. Total soluble phenolic content (TSP), phenolic acid profiles, total antioxidant activity (TA) and in vitro inhibitory activities of hyperglycemia relevant α -amylase and α -glucosidase enzymes of the dark germinated barley sprouts were evaluated at day 2, 4, and 6 post elicitor treatments. Overall, TSP content, TA, and α -amylase inhibitory activity of dark germinated barley sprouts decreased, while α -glucosidase inhibitory activity and gallic acid content increased from day 2 to day 6. Among barley cultivars, high phenolic antioxidant-linked anti-hyperglycemic bioactives were observed in Celebration. Furthermore, GP and COS seed elicitor treatments in selective doses

improved T2D relevant phenolic-linked anti-hyperglycemic bioactives of barley sprouts at day 6. Therefore, such seed elicitation approach can be strategically used to develop bioactive enriched functional food ingredients from cereal sprouts targeting chronic hyperglycemia and oxidative stress linked to T2D.

Keywords Antioxidants · Elicitors · Phenolic bioactives · Sprouting · Type 2 diabetes

Introduction

Type 2 diabetes (T2D), a non-communicable chronic disease is currently among the most significant global public health challenges (Stumvoll et al. 2005). The pathogenesis and progression of this disease is directly linked to chronic hyperglycemia and chronic oxidative stress state (Kahn 2003). Therefore regulation of postprandial absorption of glucose in the small intestine, by modulating the activities of carbohydrate solubilizing enzymes such as α -amylase and α -glucosidase is key strategy for controlling chronic hyperglycemia linked to T2D (Nair et al. 2013). Current pharmaceutical strategies involve the use of synthetic inhibitors to regulate the activity of these enzymes, to delay starch digestion, and reduce the rate of glucose absorption at the brush border cells of the gut (Hanhineva et al. 2010; Inzucchi et al. 2015). However, such drugs have been associated with side effects such as severe gastrointestinal distress and hepatic damage and therefore a wider search for safer oral hypoglycemic agents from natural and commonly used food sources using in vitro models has merit (Ali Asgar 2013; Kwon et al. 2006, 2007).

Phenolic metabolites from plant food sources have shown inhibitory activity against α -amylase and α -

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glucosidase using *in vitro* models, and have potentially minimal side-effects (Fowler 2008; Kwon et al. 2006; Stumvoll et al. 2005; Tundis et al. 2010). Being potent antioxidants, these compounds may offer an additional level of protection against the detrimental effects of oxidative stress commonly associated with chronic hyperglycemia. Therefore, the development of diversity of phenolic bioactive enriched plant-based food matrices as part of improved diets that can potentially aid in mitigating chronic hyperglycemia and oxidative stress linked to T2D holds significant merit (Kähkönen et al. 1999).

These health relevant phenolic bioactives and associated functionalities can be enhanced in plant systems by stimulating protective endogenous pathways such as redox-linked pentose phosphate pathway (PPP) (Shetty and Wahlqvist 2004). Previous published studies have demonstrated that natural elicitor treatments, in conjunction with sprouting can be used to stimulate such PPP associated endogenous defense responses and subsequent increase in phenolic bioactives and antioxidant enzyme responses in different plant models (Andarwulan and Shetty 1999; Randhir et al. 2004, 2009). Among the various natural elicitors, chitosan oligosaccharide (COS) and marine protein hydrolysate were selected in this current study, due to their effectiveness, relatively low cost, and compatibility to use in common agricultural practices. Chitosan oligosaccharides, derived via the deacetylation of marine chitin, are capable of inducing pathogen and wound stress associated molecular pattern signal cascades, which include elevated synthesis of phenolic compounds and phytoalexins (Sarkar and Shetty 2014; Trouvelot et al. 2014). Marine protein hydrolysate, are derived from proteolysis of fish and seaweed processing byproducts. It has been shown to influence the activity of enzymes of the TCA cycle, and interactions between carbon and nitrogen metabolic pathways (du Jardin 2015). Furthermore, it can also upregulate the PPP resulting in over expression of phenolic metabolites and endogenous antioxidant enzyme responses (Sarkar and Shetty 2014).

Therefore seed elicitor treatments can be strategically targeted in cereal sprouts to enhance health relevant phenolic bioactives and associated functionalities by upregulating protective PPP pathway. Sprouting is a simple and cost-effective strategy capable of enhancing the overall nutritional qualities including health relevant functionalities of cereal and legumes by breaking down anti-nutrients, improving digestibility, and availability of amino acids, soluble carbohydrates and dietary fiber (Hübner and Arendt 2013). Moreover, phenolic bioactives of sprouts may be improved due to solubilization of bound phenolics, and synthesis of phenolics as a protective mechanism to cope

with elevated oxidative stress, and for structural adjustments which is important during early stages of germination and emergence. While most of the barley under current production is utilized for malting and brewing, and feed purposes, interest in barley as a food grain source of health relevant phytochemicals is steadily growing (Baik and Ullrich 2008).

The abundance and diversity of phenolic compounds in barley also makes it unique source of beneficial phenolic bioactives among the major cereal grains. Additionally, barley grains have a high, uniform rate of germination and are physiologically well adapted for controlled germination and malting (Baik and Ullrich 2008; Hübner and Arendt 2013). Thus, barley grains were targeted to evaluate a seed elicitor-mediated approach for improvement of phenolic bioactives and associated T2D relevant anti-hyperglycemic functionalities. The major objective of this study was to evaluate the efficacy of marine protein hydrolysates (GroPro[®]; GP) and chitosan oligosaccharide (COS) as seed elicitor treatments to stimulate health relevant phenolic biosynthesis in dark germinated barley sprouts. Additionally, dark germinated barley sprouts were also evaluated at early emergence stages (i.e. 2, 4 and 6 days post seed treatments) for their phenolic antioxidant-linked anti-hyperglycemic and antioxidant functions linked to early stages of T2D, using *in vitro* assay models.

Materials and methods

Materials

All reagents and enzymes used in this study were purchased from Sigma Aldrich Chemical Co. (St Louis, MO), unless otherwise mentioned.

Preparation of elicitor treatment solutions

Two types of bioprocessed elicitors were evaluated in the current study—marine protein hydrolysate (GroPro/GP; derived from seaweed and marine fish extracts) obtained from Icelandic Bio-Enhancers (Westchester, NY, USA) and soluble chitosan oligosaccharide (derived from shells of marine crustaceans) cross linked with ascorbic acid, obtained from Kong Poong Bio (Jeju, South Korea). Both elicitors were dissolved in distilled water separately to obtain solutions of the following concentrations—GP1 & COS1 (1 g/L), GP2 & COS2 (2 g/L), GP5 & COS5 (5 g/L) and GP10 & COS10 (10 g/L). Thus, a total of 8 different elicitor treatments were evaluated, and distilled water was used as a control treatment.

Seed priming and dark germination

Seeds of 2 malting barley cultivars (Pinnacle—2 row; Celebration—6 row) were obtained from the Malting Barley Breeding Program at North Dakota State University (Fargo, ND). The seeds were disinfected in 0.5% sodium hypochlorite solution for 5 min, rinsed thoroughly with distilled water and the excess water was blotted out using paper towels. The seeds were then transferred to conical flasks containing 150 mL of elicitor treatment solution and incubated on a rotary shaker for 8 h at 20 °C and 150 rpm.

Post incubation, the seeds were rinsed with distilled water and blotted out the excess water using paper towels. Treated seeds were placed in sterile perforated plastic flats lined with moist paper towels, covered with aluminum foil to simulate dark conditions and sprouted at room temperature (20–24 °C). Germination was monitored daily, and sprouts were moistened regularly to prevent desiccation. Dark germinated sprouts were assayed on day 2, 4 and 6 post seed elicitation treatments.

Sample extraction

Dark germinated barley sprouts were excised to exclude rootlets, and homogenized in pre-chilled distilled water (3 g in 30 mL) using a laboratory blender (Waring, Torrington, CT, USA). The homogenate was centrifuged first at 8500 rpm for 20 min, followed by a second round of centrifugation at 8500 rpm for 15 min. The resulting supernatant was stored at 4 °C for in vitro assays.

Total soluble phenolic content

Total soluble phenolic (TSP) content of the dark germinated sprout extracts was determined using a modified Folin–Ciocalteu (FC) method (Shetty et al. 1995). Sample extracts (1 mL) were combined with 95% ethanol (1 mL), distilled water (5 mL), 50% v/v FC reagent (500 µL), and 5% w/v Na₂CO₃ (1 mL) in a test tube, vortexed, and incubated in the dark for 60 min. The absorbance of the resultant reaction mixtures was measured at 725 nm using a Genesys 10S UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then TSP content of the samples was calculated using a gallic acid standard curve (10–300 µg/mL; dissolved in 95% ethanol) and expressed as milligram equivalents of gallic acid (GAE) per gram fresh weight (FW).

Determination of major phenolic compounds using HPLC

Sample extracts were centrifuged for 10 min at 13,000 rpm and subjected to chromatographic analysis using an Agilent

1260 Infinity Series reverse phase HPLC, equipped with DAD 1100 diode array detector (Agilent Technologies, Palo Alto, CA). A gradient elution method, involving 10 mM phosphoric acid (pH 2.5; Solvent A) and 100% methanol (Solvent B), was used. Sample extracts were eluted on a C-18 analytical column (Agilent Supelco SB-C18 250 × 4.6 mm internal diameter) with a packing material particle size of 5 µm, at a flow rate of 0.7 mL/min at ambient temperature, with a total run time of 25 min. Pure standards of phenolic acids in 100% methanol were used to calibrate retention times on the standard curve. Chromatograms obtained were then analyzed using Agilent Chemstation integration software. Phenolic acid concentrations were expressed in micrograms per gram FW of barley sprouts.

DPPH free radical scavenging assay

The total antioxidant activity was measured using a modified DPPH (2,2 diphenyl-1-picrylhydrazyl) free radical scavenging assay (Cervato et al. 2000). A working solution of DPPH was prepared by diluting a 10 mM stock solution with 95% ethanol such that its absorbance was adjusted to a range of 1.8–2.0 at 517 nm. Barley sprout extracts (250 µL) were combined with DPPH working solution (1.25 mL), mixed thoroughly and incubated in a dark for 5 min. Following this, the absorbance of the reaction mixture was measured at 517 nm. For each sample, a corresponding control was prepared by replacing the sample with 95% ethanol (250 µL). Standard solutions of ascorbic acid dissolved in distilled water (50, 75, 125, 250, 500 and 1000 µg/mL) were used as a positive control. The DPPH free radical scavenging capacity of barley sprout extracts was calculated as per the equation:

$$DPPH \text{ inhibition } (\%) = \frac{(Abs \text{ control} - Abs \text{ sample})}{Abs \text{ control}} \times 100$$

ABTS free radical scavenging assay

The antioxidant activity of barley sprout extract was also measured by the ABTS⁺ free radical cation-decolorization assay (Re et al. 1999). A stock solution of ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) was prepared by combining 7 mM ABTS (5 mL) with 140 mM potassium persulphate (88 µL). The mixture was incubated for at least 16 h prior to the assay. The stock solution was then diluted with 95% ethanol to prepare a working solution with an absorbance of 0.70 ± 0.02 at 734 nm. Barley sprout extract (50 µL) was added to the working solution (1 mL), mixed on a vortex mixer for 30 s and incubated for 2.5 min at room temperature in a dark cabinet. Absorbance

values of the reaction mixtures were measured at 734 nm. For each sample, a corresponding control was prepared by replacing the extract with 95% ethanol (50 μ L). As with the DPPH radical scavenging assay, standard solutions of ascorbic in distilled water (50, 75, 125, 250, 500 and 1000 μ g/mL) were used as a positive control for comparison. The ABTS⁺ free radical scavenging capacity of barley sprout extracts was calculated as per the equation:

$$\text{ABTS inhibition (\%)} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100$$

Oxygen radical absorbance capacity (ORAC) assay

Oxygen radical absorbance capacity (ORAC) of dark germinated barley sprouts was determined using an assay modified from Huang et al. (2002). Zen Bio (Zen Bio Inc. Research Triangle Park, NC, USA) antioxidant assay kit was used to determine the ORAC values of barley spouts. A stock solution of fluorescein (4.19×10^{-3} mM) was prepared in 75 mM potassium phosphate buffer (PBS; pH 7.4) and stored at 4 °C until further use. Prior to the performing the assay, the stock solution was diluted with PBS to produce a working solution (8.16×10^{-5} mM). A solution of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH; 153 mM) was prepared.

Briefly, 25 μ L of barley extract (or trolox standards), 150 μ L of fluorescein working solution diluted in 75 mM phosphate buffer, pH 7.4 were pipetted into the well of microplate. The mixture was pre-incubated for 10 min at 37 °C, and then 25 μ L of 153 mM AAPH were added rapidly using a multichannel pipette. The fluorescence ($\lambda_{\text{ex}} = 485$ nm; $\lambda_{\text{em}} = 520$ nm) was recorded every minute for 30 min using BioTek Synergy H1 Hybrid Multimode fluorescence reader (BioTek Instruments, Inc., Winooski, VT, USA). Calibration solutions of Trolox (100, 50, 25, 12.5 and 6.25 μ M) were also carried out in the assay. Data were exported and area under the curve (AUC) and net area under the curve of sample were calculated by subtracting blank AUC value and were plotted against Trolox standard net AUC. The ORAC values of the barley were expressed as μ mole Trolox equivalents (TE) per gram of sample.

α -Amylase inhibitory activity

The α -amylase inhibitory activity was determined by an assay utilized by Kwon et al. (2006). Sprout extracts (500 μ L) were mixed with 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M NaCl; 500 μ L) containing porcine pancreatic α -amylase (0.5 mg/mL) and incubated at 25 °C for 10 min. Starch solution (1% w/v, prepared in

0.02 M sodium phosphate buffer; 500 μ L) was added and further incubated at 25 °C for 10 min. Finally, 3,5-dinitrosalicylic acid (1 mL) solution was added and the reaction was stopped by incubating the mixtures in a boiling water bath (90–100 °C) for 10 min. Reaction mixtures were cooled to room temperature and diluted with distilled water to bring control absorbance readings within a range of 0.8–1.0 units at 540 nm. Sample blank (containing sodium phosphate buffer instead of enzyme solution) absorbance and a control (containing sodium phosphate buffer in instead of sprout extract) were also recorded. The α -amylase inhibitory activity was calculated as percentage (%) inhibition per the equation:

$$\alpha\text{-Amylase inhibition (\%)} = \frac{(\text{Abs control} - (\text{Abs sample} - \text{Abs sample blank}))}{\text{Abs control}} \times 100$$

Sample results were compared with Acarbose positive control (0.05, 0.07, 0.12, 0.25, 0.50 and 1.0 mg/mL).

α -Glucosidase inhibitory activity

The α -glucosidase inhibitory potential was determined using an assay modified from the Worthington Enzyme Manual (Kwon et al. 2006). Sprout extracts (10, 25, 50 μ L) were loaded in 96-well microtiter plates, and the volume in each well was made up to 100 μ L with 0.1 M phosphate buffer (pH 6.9). To each well, α -glucosidase solution (1 unit/mL, prepared in 0.1 M phosphate buffer; 100 μ L), was added, and the plate was incubated at 25 °C for 10 min. Then, 5 mM *p*-nitrophenyl- α -D-glucopyranoside (pNPG; 50 μ L) solution was added to all wells, and the plate was incubated at 25 °C for 5 min. Absorbance values were recorded before and after incubation at 405 nm using a microplate reader (Thermomax, Molecular Device Co., Sunnyvale, CA) and compared to a corresponding control containing buffer solution (50 μ L) instead of sample extract. The α -glucosidase inhibitory activity was expressed as percentage (%) inhibition and was calculated per the equation:

$$\alpha\text{-Glucosidase inhibition (\%)} = \frac{(\Delta \text{Abs control} - \Delta \text{Abs sample})}{\Delta \text{Abs control}} \times 100$$

Sample results were compared with Acarbose positive control (0.05, 0.07, 0.12, 0.25, 0.50 and 1.0 mg/mL).

Statistical analysis

A completely randomized design (CRD) was used for this study. For each sample, six replicates were analyzed during each run of the assays, and the experiment was repeated

twice. Calculation of means and standard deviations, along with Pearson's correlation coefficient (PCC, r) was performed using Microsoft Excel 2016. Analysis of variance (ANOVA) for the data was performed using the Statistical Analysis Software (SAS; version 9.4; SAS Institute, Cary, NC). Statistical differences in bioactive functionality parameters, due to differences in elicitor treatments, cultivars, and the interaction between cultivars $\times$ treatments were determined using the Tukey's least mean square test at a confidence level of 95% ($p < 0.05$).

Results and discussion

Total soluble phenolic content

The total soluble phenolic (TSP) content of dark germinated barley sprouts after seed elicitation treatments was measured using the Folin–Ciocalteu method. Overall, the TSP content of the dark germinated barley sprouts across cultivars and elicitor treatments ranged from 0.33 to 0.76 mg/GAE g⁻¹ FW and it decreased with the progression of the sprouting from day 2 to day 6 of sprouting (Fig. 1a–c). This TSP range when corrected on DW basis was higher than baseline TSP content of 0.45–0.5 mg/GAE g⁻¹ DW of flour derived from same cultivars (Ramakrishna et al. 2017). Such higher TSP content in the barley sprouts was likely due to the mobilization of free and bound phenolics coupled with new synthesis in response to seed priming and elicitation. Ha et al. (2016) found phenolic content in primed barley sprouts in the range of 1.06–3.37 mg/GAE g⁻¹ DW. Further the same study had similar trends in modulation of TSP content in barley sprouts primed with distilled water, and observed that TSP initially increased at 48 h post soaking and then sharply decreased subsequently. The decrease in phenolic content with progression of sprouting might be due to the potential utilization of phenolic compounds in the mobilization processes during seed germination (Randhir et al. 2004). In this study, significant differences in TSP content of dark germinated barley sprouts were observed between cultivars, treatments, and cultivar $\times$ treatment interactions at day 2 and day 6 of sprouting. At day 2 and day 6, dark germinated sprouts of Celebration had significantly higher TSP content when compared to Pinnacle ($p < 0.05$).

The highest TSP content was found in dark germinated sprouts of Celebration with elicitor treatments GP5, GP10, COS 1, and COS 5 at day 2 and day 4 of sprouting. However, it was statistically at par with the control ($p < 0.05$) (Fig. 1a, b). At day 6, GroPro (GP5 & GP10) and COS (COS2 & COS5) elicitor treatments resulted in significantly higher TSP content in Celebration when compared to the control ($p < 0.05$) (Fig. 1c). Based on the

TSP content results of this study, Gro-Pro (5 & 10 g/L) and COS (2 g/L) can be targeted to improve phenolic bioactive content of barley and other grain or legume sprouts in the future. Previously, Randhir et al. (2009) reported that fish protein hydrolysates (2 mL/L), with a composition similar to Gro-Pro improved TSP content in dark germinated mung bean (*Vigna radiata*) sprouts during the initial stages of germination. Therefore, this seed elicitation strategy with bioprocessed elicitor such as Gro-Pro and COS has significant merit to improve human health relevant phenolic bioactives and such strategy can be used for developing phenolic-enriched functional food ingredients in other grains and legumes.

Phenolic acids profile

The major phenolic acids in the elicited, dark germinated barley sprout extracts were determined using reverse phase HPLC method. The major phenolic acids detected in this study were catechin, gallic acid, protocatechuic acid, and dihydroxybenzoic acid (Table 1). The concentration of gallic acid was found to be the highest in two barley cultivars and across all treatment types. Although TSP content of barley sprouts decreased with the progression of the sprouting, the gallic acid content increased during sprouting and it was more evident in Pinnacle. At day 2 and day 6 of sprouting, GP1 elicitor treatment resulted in higher gallic acid content in both barley cultivars when compared to the control and other elicitor treatments. Gallic acid content of food has relevance for managing oxidative stress and other metabolic disorders commonly associated with chronic hyperglycemia (Lee et al. 2015). Inhibition of proinflammatory cytokine production in human monocytes in the presence of gallic acid was observed previously (Lee et al. 2015). Addition (50%) of gallic acid in proportion with Acarbose (50%) also resulted in higher α -glucosidase inhibitory activity (Obboh et al. 2016). In this study both gallic acid content (Table 1) and α -glucosidase inhibitory activity (Table 4) increased from day 2 to day 6 which indicated a potential relevance of gallic acid for inducing α -glucosidase inhibitory activity in these dark germinated barley sprouts. Significant improvement in catechin content was also observed with Gro-Pro and COS elicitor treatments (GP5, GP10, COS5) in Celebration at day 2, 4, and 6 of sprouting. Catechin is a known antioxidant with potential anti-hypertensive, anti-inflammatory, and anti-proliferative functions (Babu and Liu 2008; Iacopini et al. 2008). Therefore, improving catechin content (Table 1) through elicitor treatment in dark germinated barley sprouts may have significant relevance against oxidative stress-induced macro-vascular complications which is commonly associated with early stages T2D. Overall, higher concentration of dihydroxybenzoic acid was found in Pinnacle, while

Fig. 1 Total soluble phenolic (TSP) content (mg GAE/g fresh weight) of dark germinated barley sprouts of two cultivars, measured on day 2 (**a**), day 4 (**b**) and day 6 (**c**) after priming with elicitor treatments (chitosan oligosaccharide-COS & marine hydrolysate-GP). Different alphabets represent significant differences in TSP content due to cultivar $\times$ treatment interactions at 95% confidence level ($p < 0.05$) separately for each day of sprouting

Table 1 Dominant phenolic acids in dark germinated barley sprouts 2 days (a), 4 days (b) and 6 days (c) post priming with seed elicitor treatments

Treatment	Average concentration ($\mu\text{g/g}$ FW)							
	Catechin		Gallic acid		Protocatechuic acid		Dihydroxybenzoic acid	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
<i>a</i>								
Control	1.13a	0.40a	0.75f	7.36cde	n.d.	n.d.	n.d.	0.41cd
GP1	0.42a	0.64a	6.72cde	10.0bcd	n.d.	0.62d	9.43a	4.52abcd
GP2	0.36a	0.62a	5.52def	9.21bcde	n.d.	0.30e	9.28a	0.50cd
GP5	0.37a	0.59a	5.15def	7.36cde	n.d.	0.53d	1.19cd	0.41cd
GP10	0.30a	0.70a	4.98def	9.11bcde	n.d.	1.07b	8.19cd	0.48cd
COS1	0.38a	0.71a	4.96def	8.26bcde	n.d.	0.18ef	7.38a	1.06cd
COS2	0.35a	0.55a	5.49def	15.4a	n.d.	1.61a	5.97abc	1.53bcd
COS5	0.29a	0.57a	4.56ef	12.7ab	n.d.	0.84c	0.67cd	0.34cd
COS10	0.38a	0.59a	6.32cde	11.0abc	n.d.	1.44a	7.09ab	1.45bcd
<i>b</i>								
Control	0.34j	0.48e	14.7a	12.5cde	n.d.	1.48a	5.66a	0.69a
GP1	0.43g	0.48ef	14.1ab	12.0cdef	n.d.	1.15a	4.22a	0.45a
GP2	0.31	0.58ab	11.5def	13.2bc	n.d.	0.66a	0.86a	0.48a
GP5	0.54cd	0.57abc	13.0bc	10.9wf	n.d.	1.05a	1.05a	0.45a
GP10	0.45fg	0.59a	13.0bc	12.5cd	0.20a	1.66a	1.25a	0.48a
COS1	0.39i	0.43gh	11.1ef	11.4def	0.18a	0.97a	0.99a	0.56a
COS2	0.40j	0.60a	12.4cde	12.1cdef	1.35a	0.98a	0.60a	0.41a
COS5	0.55bcd	0.54d	11.8cdef	9.3g	1.31a	0.55a	1.16a	0.42a
COS10	0.46efg	0.53d	12.3cdef	12.2cdef	0.16a	0.89a	0.76a	0.40a
<i>c</i>								
Control	0.34def	0.25gh	12.4a	12.5a	0.15fgh	n.d.	0.78a	0.46d
GP1	0.50b	0.22h	16.2a	17.7a	0.04h	1.22a	0.70abc	0.40d
GP2	0.30efg	0.34def	14.6a	13.3a	0.34def	0.34def	0.73ab	0.41d
GP5	0.34def	0.35cdef	14.2a	8.81a	0.07gh	0.87h	0.54bcd	0.43d
GP10	0.08i	0.50b	12.4a	17.2a	0.12fgh	0.59cd	0.48cd	0.45d
COS1	0.35def	0.29fg	14.5a	15.2a	0.22fgh	0.31efg	0.54bcd	0.43d
COS2	0.59a	0.41c	14.1a	1.62a	0.33def	0.51cde	0.87bcd	0.41d
COS5	0.20h	0.40cd	11.5a	14.1a	0.05h	0.61c	0.34d	0.39d
COS10	0.37cde	0.19h	9.00a	14.6a	0.15fgh	0.99ab	0.32d	0.35d

n.d. not detected

higher protocatechuic acid was observed in Celebration. Similar to gallic acid, protocatechuic acid is also potent antioxidant with diverse human health relevant and pharmacological functions (Kakkar and Bais 2014). Higher in vitro antioxidant activity of protocatechuic acid compared to Trolox was reported previously (Li et al. 2011). Higher antioxidant activity of Celebration might be associated with the higher concentration of protocatechuic acid (Table 1) in this barley cultivar when compared to Pinnacle (not detected at day 2 and day 4). Therefore, the phenolic acid profile and the response of barley to seed elicitation

treatments may be dependent on the cultivar or cultivar $\times$ environment interactions.

However, while ferulic acid has been reported as the most dominant phenolic acid in previous studies involving barley grains, it was not detected in the dark germinated sprout extracts of this study (Dykes and Rooney 2007). Various factors may have contributed for not finding ferulic acid in this study, such as the use of dietary relevant aqueous extracts, the choice of mobile phase, and HPLC protocols used in this study. However, improvement of gallic acid, catechin, and protocatechuic acid in

Celebration with seed elicitation treatments has significant relevance and such strategy can be used to enhance targeted human health relevant phenolic bioactives in barley or in other grain sprouts.

Antioxidant activity and antioxidant capacity

The antioxidant activity of the dark germinated barely sprout extracts was evaluated using DPPH and ABTS free radical scavenging based assays. Furthermore, ORAC values of dark germinated barley sprout extracts were also measured. Overall, significant differences in total antioxidant activity (based on both DPPH and ABTS assay) of dark germinated barley sprouts were observed between cultivars, treatments, and cultivar $\times$ treatment interactions ($p < 0.05$) at all stages of the sprouting (Table 2b, c). However, significant difference in antioxidant capacity (ORAC) was only observed between the two barley cultivars at day 2 and day 4 (Table 2a). Dark germinated sprout of Celebration had significantly higher antioxidant capacity when compared with Pinnacle at day 2. Like the TSP content and antioxidant capacity (based on ORAC dark germinated sprouts of Celebration had significantly ($p < 0.05$) higher antioxidant activity (DPPH & ABTS) than Pinnacle at all three sprouting stages (Table 2b, c). When compared with ascorbic acid, DPPH inhibition of Celebration (average TSP of 70.0 $\mu\text{g/mL}$) at day 2 was equivalent to 350 $\mu\text{g/mL}$ ascorbic acid, while for Pinnacle (average TSP of 45.0 $\mu\text{g/mL}$) it was 200 $\mu\text{g/mL}$ ascorbic acid. However, at day 6 the DPPH % inhibition of both cultivars (average TSP of 37.0 and 35.0 $\mu\text{g/mL}$ for Celebration and Pinnacle) was equivalent to 100–120 $\mu\text{g/mL}$ ascorbic acid. Similarly, antioxidant activity based on ABTS % inhibition for Celebration (70.0 $\mu\text{g/mL}$ TSP) and Pinnacle (45.0 $\mu\text{g/mL}$ TSP) at day 2 was equivalent to 50 and 35 $\mu\text{g/mL}$ ascorbic acid. The antioxidant activity results indicated better response of barley extracts and ascorbic acid towards ABTS radical scavenging assay when compared with DPPH radical scavenging assay as reported previously (Re et al. 1999). While, Celebration sprouts treated with GP5 were found to have the highest antioxidant activity based on the DPPH assay at day 2 (22.45%) and day 4 (13.65%), however significant difference ($p < 0.05$) with control was only observed at day 2 (Table 2b). Further, at day 6, GP2, COS1, and COS2 seed elicitation treatment resulted in significantly higher antioxidant activity (based on DPPH) in Celebration and this result corroborated with TSP content of the same cultivar. Overall, antioxidant activity (based on DPPH and ABTS) and antioxidant capacity (based on ORAC) was found to steadily decline over the course of sprouting and this trend is consistent with that of decreasing TSP content at the same stages. A strong positive correlation was found

between TSP content and DPPH free radical scavenging capacity at day 2 ($r = 0.96$).

Similar trend was observed in dark germinated mung bean (Randhir et al. 2009) and in barley sprouts (Ha et al. 2016), where the antioxidant activity peaked during the first 24–48 h of germination. However, this can be attributed to an increased demand for oxygen for respiration by sprouts in the initial stages of emergence (Randhir et al. 2009). Further, total antioxidant activity of the barley sprouts decreased in tandem with declining TSP content, which indicates the significant antioxidant function of soluble phenolic compounds in the barley sprouts. Overall, higher total antioxidant activity of dark germinated barley sprouts was observed based on ABTS free radical scavenging assay and the differences between DPPH and ABTS based assays are potentially linked to the water soluble phenolic fractions responsible for radical scavenging activity, and which are generally more responsive to the ABTS assay (Re et al. 1999). Dark germinated sprouts of Celebration with COS2 treatment had highest antioxidant activity based on ABTS free radical scavenging assay at day 2, however was at par with the control and other elicitor treatments (Table 2c). At day 4, Pinnacle treated with GP1 had significantly higher ($p < 0.05$) total antioxidant activity (based on ABTS) when compared to the control (Table 2c). Similarly, at day 6 dark germinated barley sprouts of Celebration treated with GP5 had significantly ($p < 0.05$) higher total antioxidant activity (based on ABTS) when compared to the control (Table 2c).

Like the DPPH based antioxidant results, strong and positive correlations between TSP content and ABTS based antioxidant activity ($r = 0.82$) was observed at day 2 of the sprouting. The correlation between two antioxidant assays (DPPH and ABTS) was also found to be higher and positive at day 2 ($r = 0.77$) and day 4 ($r = 0.48$) of sprouting. These results suggest that total antioxidant activity of barley sprouts was closely linked with the TSP content. Therefore, enhancement of TSP content might result in the improvement of antioxidant functions of barley sprouts which has potential relevance for targeting multi-functional dietary therapy of chronic hyperglycemia-linked oxidative stresses commonly associated with early stages of T2D.

α -Amylase and α -glucosidase inhibitory activity

To evaluate anti-hyperglycemic functions of dark germinated barley sprouts, relevant α -amylase and α -glucosidase enzyme inhibitory activities were determined using rapid in vitro assay models. Overall, like the TSP content and total antioxidant activity, α -amylase inhibitory activity of dark germinated barley sprouts also decreased with the progression of the sprouting from day 2 to day 6 (Table 3a–c). Similarly, Randhir et al. (2009) found higher

Table 2 Antioxidant capacity of dark germinated barley sprouts, determined by ORAC assay (a, μM of Trolox Equivalent), DPPH radical inhibition assay (b, %) and ABTS radical scavenging assay (c, %)

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
<i>a</i>						
Control	66.2b	70.9b	54.2a	58.6a	48.7a	46.7a
GP1	64.2b	91.5ab	56.7a	58.4a	49.6a	48.9a
GP2	70.2b	68.8b	58.9a	61.2a	44.6a	52.4a
GP5	68.9b	86.6ab	55.6a	62.4a	46.8a	50.4a
GP10	61.2b	67.6b	58.4a	60.4a	43.2a	55.6a
COS1	65.6b	84.7ab	59.7a	56.6a	44.5a	48.4a
COS2	69.2b	73.9b	54.2a	56.8a	48.9a	49.7a
COS5	71.2b	114.1a	52.6a	50.6a	49.4a	46.2a
COS10	68.9b	72.8b	53.7a	52.7a	46.6a	50.1a
<i>b</i>						
Control	11.7de	19.3bc	5.15fg	12.0ab	5.60de	8.82c
GP1	11.0e	20.8ab	5.75efg	9.29bcd	6.59d	9.41bc
GP2	7.05f	20.6ab	5.54efg	6.64defg	4.82de	12.1a
GP5	7.28f	22.4a	4.87fg	13.6a	5.82de	9.22bc
GP10	8.87ef	20.5ab	5.08fg	10.8bc	4.33e	9.45bc
COS1	8.88ef	20.6ab	4.71fg	9.08cd	4.50e	11.1ab
COS2	7.11f	17.6c	4.24g	8.01de	6.63d	10.8ab
COS5	6.78f	18.7bc	4.85fg	9.33bcd	5.79de	10.1abc
COS10	7.63f	14.1d	5.64efg	7.29def	6.14de	9.55bc
<i>c</i>						
Control	14.6g	37.8ab	12.3cde	23.3ab	12.5c	15.4bc
GP1	26.3cdef	21.7sdefg	26.5a	19.2abc	12.8bc	16.8bc
GP2	19.8defg	29.9abcd	15.8bcde	17.1bcd	15.6bc	16.9abc
GP5	12.6g	27.4bcde	11.2cde	18.6abcd	13.2bc	24.3a
GP10	19.0efg	28.9bcde	8.96e	19.2abc	15.8bc	20.1ab
COS1	12.1g	32.0abc	15.3bcde	18.2bcd	16.0bc	17.1abc
COS2	14.9g	41.0a	10.6de	22.3ab	16.8abc	17.8abc
COS5	12.4g	30.8abcd	18.6abcd	20.8ab	19.8abc	18.4abc
COS10	16.0fg	27.5bcde	21.3ab	18.7abc	20.1ab	16.6bc

In each assay, different alphabets represent significant difference due to cultivar $\times$ treatment interactions at 95% confidence level ($p < 0.05$), separately for each day of sprouting

α -amylase inhibitory activity of mung bean sprout at day 1 than day 4 following seed elicitation treatments. However, completely opposite trend was observed for α -glucosidase, as inhibitory activity against this key targeted enzyme increased steadily from day 2 to day 6 (Table 4a–c). Further for both enzyme inhibitory activities, significant dose dependent response was also observed in dark germinated sprouts of two barley cultivars. Overall, significant differences in α -amylase and α -glucosidase enzyme inhibitory activities of barley sprouts due to differences in cultivars, treatments, and cultivar $\times$ treatment interactions were observed in all three stages of the sprouting ($p < 0.05$). For all dilutions (undiluted, half, and one-fifth) and across all treatments significantly ($p < 0.05$) higher α -amylase inhibitory activity was observed in Celebration at day 2,

however Pinnacle had significantly higher α -amylase inhibitory activity at day 4 and day 6 (Table 3a–c). When compared with the positive control, the α -amylase inhibitory activity of Celebration (100 mg/mL) at day 2 was equivalent to 1–1.2 mg/mL of Acarbose, however for Pinnacle (100 mg/mL) it was equivalent to 0.25 mg/mL of Acarbose. Similar to the TSP content and antioxidant activity of Celebration, α -amylase inhibitory activity decreased significantly with the progression of the sprouting and this result suggests that the α -amylase inhibitory activity might be linked to the TSP content of the barley sprouts. Positive and moderate correlations between TSP content and α -amylase inhibitory activity was observed at all three stages of the sprouting ($r = 0.66; 0.59; 0.39$ at day 2, 4, and 6 respectively).

Table 3 α -Amylase inhibitory activity (%) of undiluted (a), 1-in-2 part (b) and 1-in-5 part diluted (c) dark germinated barley sprout extracts

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
<i>a</i>						
Control	68.9c	91.0ab	75.6cd	51.1e	37.9defg	27.9fgh
GP1	86.6ab	91.8ab	73.8cd	63.0e	45.1abcd	12.0i
GP2	77.0bc	94.0a	47.0fgh	33.9h	54.6ab	13.1hi
GP5	66.4c	94.8a	63.7de	44.0gh	58.4a	31.5efg
GP10	28.7e	89.3ab	55.6ef	89.0ab	44.2abcde	14.1hi
COS1	87.5ab	92.0a	75.5dc	82.2abc	44.0abcdefg	37.4cdefg
COS2	46.3e	86.7ab	91.0ab	94.8a	29.6efg	29.5gh
COS5	87.7ab	87.3ab	45.1fgh	75.0bc	52.3abc	41.3abcdef
COS10	63.8c	91.3a	86.7abc	45.3fgh	43.9bcdefg	43.4abcdef
<i>b</i>						
Control	58.8cdef	82.6a	56.9a	13.4fg	22.1bcd	0.34e
GP1	53.6efg	74.1abc	42.7c	49.0abc	36.7a	2.24e
GP2	55.8defg	78.1ab	40.3cd	10.4g	37.0ab	0.27e
GP5	41.3h	76.5b	53.2ab	12.0fg	32.0ab	11.7de
GP10	15.3i	79.4ab	37.7d	57.0a	33.5ab	0.95e
COS1	54.9defg	65.7bcde	43.9bc	39.0cd	26.8abc	31.5ab
COS2	36.3h	36.9h	42.9bc	56.8a	24.1bc	14.5cd
COS5	42.5fgh	71.8abc	23.4ef	55.1a	22.4bcd	17.2cd
COS10	47.1fgh	71.4abcd	48.5abc	29.8de	13.2e	26.8abc
<i>c</i>						
Control	29.4fghi	75.0a	34.2a	3.2efg	8.07bc	n.d.
GP1	19.0hij	59.4bc	20.9bcd	15.8cde	24.6a	n.d.
GP2	33.0efgh	63.7ab	11.5defg	0.76fg	13.6ab	n.d.
GP5	34.9efg	65.2ab	14.7cdef	0.00g	21.6a	n.d.
GP10	10.7j	47.6cd	19.9bcd	31.7ab	13.3ab	0.24c
COS1	36.9ef	51.2cd	10.5cdefg	18.5cd	4.65bc	11.9ab
COS2	18.1ij	16.0j	13.8cde	24.3abc	0.30c	6.27bc
COS5	14.4j	42.7de	13.3cde	21.5bcd	7.08bc	6.19bc
COS10	22.6ghij	34.8ef	15.9cde	15.9bcd	4.13bc	n.d.

Different alphabets represent significant differences in α -amylase inhibitory activity due to cultivar $\times$ treatment interactions at 95% confidence level ($p < 0.05$) separately for each day of sprouting
n.d. not detected

In this study, seed elicitation treatments also resulted in higher α -amylase inhibitory activity in dark germinated barley sprouts. In undiluted sample, COS1 treated sprouts of Pinnacle had significantly higher α -amylase inhibitory activity when compared to the control at day 2 of sprouting ($p < 0.05$) (Table 3a). However, at day 4, COS2 and at day 6, GP5 resulted in significantly higher α -amylase inhibitory activity in undiluted sample of dark germinated sprouts of Pinnacle. Therefore, these seed elicitor treatments can be targeted selectively to improve α -amylase inhibitory activity of barley or for other grain and legume sprouts.

Overall, high α -glucosidase inhibitory activity was observed in undiluted sample of both barley cultivars at day 4 and day 6 of sprouting (Table 4a). Similar to the TSP

content and total antioxidant activity, significantly higher α -glucosidase inhibitory activity was observed in dark germinated barley sprouts of Celebration when compared to the Pinnacle ($p < 0.05$). α -Glucosidase inhibitory activity of undiluted sample of Celebration (100 mg/mL) at day 2 and day 6 was equivalent to 0.15 and 1 mg/mL Acarbose (positive control) respectively. Similarly, for undiluted sample of Pinnacle (100 mg/mL), α -glucosidase inhibitory activity at day 2 and day 6 was equivalent to 0.07 and 0.6 mg/mL of Acarbose. Among seed elicitor treatments, significant ($p < 0.05$) improvement in α -glucosidase inhibitory activity of dark germinated sprouts of Pinnacle was observed with COS2 and COS5 at day 4 and day 6 of sprouting. However, for dark germinated sprouts of Celebration, GP2 seed

Table 4 α -Glucosidase inhibitory activity (%) of undiluted (a), 1-in-2 part (b) and 1-in-5 part diluted (c) dark germinated barley sprout extracts. Different alphabets represent significant differences in α -amylase inhibitory activity due to cultivar $\times$ treatment interactions at 95% confidence level ($p < 0.05$) separately for each day of sprouting

Treatment	Day 2		Day 4		Day 6	
	Pinnacle	Celebration	Pinnacle	Celebration	Pinnacle	Celebration
a						
Control	47.8abcd	46.5bcd	58.6de	88.6a	72.7de	86.7ab
GP1	38.0de	49.36abcd	57.8de	88.7a	74.5cd	70.5de
GP2	42.6cde	55.9ab	61.1cd	90.9a	67.6e	92.0a
GP5	25.3f	49.9abcd	59.6cde	89.1a	79.8c	87.3ab
GP10	31.2ef	44.8bcd	56.9de	91.2a	68.6de	85.9c
COS1	22.0f	55.1ab	65.8bc	92.0a	79.2c	86.9ab
COS2	22.7f	58.8a	68.5b	90.4a	91.0ab	87.3bc
COS5	31.0ef	54.5abc	69.1b	92.1a	87.7ab	86.6bc
COS10	24.8f	50.3abc	53.2e	92.9a	70.9de	86.6bc
b						
Control	32.0bcd	31.0cde	44.5de	80.1b	57.5ef	76.5c
GP1	23.3efg	30.3cde	42.2e	80.6ab	63.0de	56.6ef
GP2	27.7def	36.7abc	46.3de	86.2ab	52.5f	86.3ab
GP5	18.4gh	32.0bcd	44.2de	80.2b	67.5d	77.9c
GP10	21.4fgh	27.3def	43.4e	86.8ab	55.6ef	78.0c
COS1	17.2gh	37.9abc	51.4cd	85.7ab	66.8d	77.3c
COS2	15.3fg	42.1a	54.3c	84.2ab	87.8a	79.5bc
COS5	18.4gh	39.3ab	55.3c	86.0ab	78.7bc	76.9c
COS10	13.2h	35.0abcd	39.4e	87.8a	56.7ef	78.9bc
c						
Control	17.2bcde	13.0ef	26.0g	64.9bcd	39.4fgh	58.70dc
GP1	11.7efg	12.4efg	24.7g	63.8cd	48.0ef	38.96fgh
GP2	14.5cdef	20.6abc	26.7g	68.7abcd	36.1h	70.78a
GP5	13.3def	17.2bcde	25.6g	62.1d	52.2de	60.72bcd
GP10	13.9def	13.4def	29.3fg	69.9abcd	35.7h	58.12cd
COS1	11.5efg	20.4abcd	31.8efg	71.6abc	47.8efg	61.60bc
COS2	9.2fg	26.4a	37.4ef	69.5abcd	69.3ab	61.87abc
COS5	5.5g	23.2ab	37.9e	73.2ab	64.5abc	57.76cd
COS10	5.56g	20.38abcd	25.01g	75.62a	39.57fgh	58.24cd

elicitor treatment resulted in significantly higher α -glucosidase inhibitory activity, especially at half and one-fifth diluted samples (Table 4b, c). Improvement of α -glucosidase inhibitory activity with COS seed elicitor treatment was also observed in black bean (*Phaseolus vulgaris*) sprouts previously (Orwat 2016). The α -glucosidase inhibitory activity of germinated barley sprouts positively correlated with the TSP content in a previous study (Ha et al. 2016). Moreover, the enzyme inhibition response was found to be dose dependent, which is essential in the design of functional food ingredients for better glycemic control (Ha et al. 2016). In this study, high and positive correlations between α -glucosidase inhibitory activity and DPPH based antioxidant activity was observed in dark germinated barley sprouts ($r = 0.82, 0.75, 0.63$ at day 2, 4 & 6). Results of this in vitro study indicated that anti-hyperglycemic and antioxidant functions of dark germinated barley sprouts are

associated with the TSP content and phenolic acids profile of barley cultivars. The use of seed elicitation strategy with safe edible elicitors has significant merit to improve overall phenolic antioxidant-linked anti-hyperglycemic functions in grain and legume sprouts and can be targeted for designing functional food ingredients to develop dietary supports against early stages of T2D.

Conclusion

Dark germination-linked sprouting in combination with seed elicitation is a simple and cost-effective strategy for improving human health relevant phenolic bioactives and associated anti-hyperglycemic and antioxidant functions in grains and legumes. However, finding suitable elicitor and optimizing effective doses is essential prior to utilizing this

metabolically-driven strategy for broader food and health relevant applications. Based on this need to improve human health relevant phenolic bioactive profiles and associated T2D relevant functionalities in dark germinated barley sprouts two different bioprocessed elicitor treatments in several doses and two different barley cultivars were strategically targeted and evaluated in this study. Overall, TSP content, antioxidant activity, α -amylase inhibitory activity and concentrations of catechin and dihydroxybenzoic acid decreased from day 2 to day 6, while α -glucosidase inhibitory activity and gallic acid content increased during sprouting stages. Seed elicitation treatments with marine protein hydrolysate (GP) and COS in selected doses induced improvement of phenolic bioactive-linked anti-hyperglycemic functions in dark germinated barley sprouts. However, the response to the seed elicitor treatment for improving human health relevant phenolic functionalities is specific to the choice of barley cultivars as higher response was observed in Celebration when compared with Pinnacle barley cultivar. Therefore, both these elicitors can be strategically targeted for improving phenolic bioactives and associated antioxidant and anti-hyperglycemic functions linked to T2D. However, further research to evaluate other barley genotypes, different grain species, and different doses of elicitor treatments are required to validate this concept for wider food design and health relevant applications and use.

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