

Effects of sprouting and salt stress on polyphenol composition and antiradical activity of einkorn, emmer and durum wheat

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Abstract

Germination is related with improvements of nutritional value of seeds, since it promotes accumulation of health-promoting phytochemicals. However, only few studies have investigated on phytochemicals accumulation during sprouting under sub-optimal conditions. Thus, we investigated the effect of salinity during germination of an einkorn (TMoM), an emmer (TDiZ) and a durum wheat (TDuC) genotype on the total polyphenols (TPC), free- and bound-phenolic acids [PAs; *i.e.* caffeic acid, syringic acid, P-coumaric acid, *trans*-ferulic acid, and salicylic acid] contents and antiradical activity (Trolox equivalent antioxidant capacity; TEAC) of sprouts and wheatgrass. The following NaCl treatments were applied: 0 (control), 25, 50 and 100 mM NaCl concentration throughout the whole experiment, or 50 and 100 mM NaCl until sprout stage and then 0 mM until wheatgrass stage (recovery treatments). TMoM showed higher total bound-PAs both in sprouts and wheatgrass with respect to the other *Triticum* genotypes (+25% and 24%, respectively) as well as of total bound-PAs and bound-SA in the recovery treatments. Moderate salt stress significantly

increased all the investigated variables in TDiZ. Salt stress induced higher TPC and TEAC as well as total free-PAs values till 50 mM NaCl in TDuC, whilst significantly lowered total bound-PAs due to the negative variation of both P-CA (-84%) and *trans*-FA (-81%) acids. Results indicate that salinity during germination could be efficiently modulated to improve the nutritional quality of sprouts, wheatgrass and cereal-based products.

Introduction

Sprouting is reported to be associated with improvements of nutritional value of seeds, since it promotes accumulation of health-promoting phytochemicals (phenolic acids, flavonoids, and vitamins) (Oh and Rajashekar, 2009).

Cereal sprouts (*i.e.* caryopses within 3-4 days after germination) and microgreens (*i.e.* young seedlings of 1-2 weeks age, known as wheatgrass) may represent a valid alternative in human feeding (Benincasa *et al.*, 2015). They allow avoiding processing treatments (*i.e.* milling and baking) thus maintaining the nutritional value of grains, mainly represented by substantial quantities of bioactive compounds – including phenolic acids – which are unevenly distributed in kernels, with aleurone layer having the highest antioxidant activity, followed by the bran fraction. The hulled wheat species, *i.e.* the diploid wheat einkorn (*Triticum monococcum* ssp. *monococcum*, genome AA) and the tetraploid emmer (*Triticum turgidum* ssp. *dicoccum*, genomes AABB), are less bred species and may be used for sprouting, to better satisfying target requirements of consumers compared to the most cultivated soft and durum wheat.

Recent works have focused on phenolic acids and flavonoids contents in *ancient* and *modern* wheat genotypes (Shewry and Hey, 2015), both on grains (Dinelli *et al.*, 2011) and germinated sprouts (Benincasa *et al.*, 2015; Hung *et al.*, 2011). However, only few studies have investigated on the response in terms of phytochemicals accumulation during sprouting under sub-optimal conditions.

The content and composition of bioactive compounds in sprouts and wheatgrass depend on many factors such as genotype, growth stage and environmental conditions during sprouting (Cevallos-Casals and Cisneros-Zevallos, 2010). It is well acknowledged that plants under various abiotic stresses (*i.e.* drought, salinity, high light and chilling; Lim *et al.* 2012; Oh and Rajashekar, 2009) accumulate several phytochemicals, which play a major role in the adaptation of plants to unfavourable growing conditions (Guo *et al.*, 2014; Yuan *et al.*, 2010). Quality traits, as well as the level and activity of bioactive compounds occurring in

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foods of plant origin may be modified using a range of biotechnological and agronomic practices. However, to date little is known about the effect of abiotic stress on phytochemical accumulation in edible sprouts and the application of environmental stress to enhance their quality traits as well as the actual translatability of the enriched-bioactive compounds to biological benefits in lifestyle-related diseases. The effect of salt stress on bioactive compounds accumulation was studied in sprouts of radish (Yuan *et al.*, 2010), broccoli (Guo *et al.*, 2014), lentil (Świeca, 2015), peanut (Kavas *et al.*, 2015), alfalfa (Wang *et al.*, 2009) and buckwheat (Lim *et al.*, 2012). In wheat (*Triticum aestivum* L.) the impact of salt stress was only evaluated in terms of germination rates and biometric determinations (Datta *et al.*, 2009) while no quality trait was assessed.

Our hypothesis is that the content of bioactive compounds of ready-to-eat sprouts and wheatgrass of *Triticum* genotypes may be enhanced by both germination process and salt stress conditions applied during sprouting. The parameters assessed to characterise these effects were total phenolic content, radical scavenging activity and the content of some phenolic acids (both free- and bound-fractions) in grains, sprouts and wheatgrass of three different *Triticum* genotypes (einkorn, emmer and durum wheat).

Materials and methods

Plant material and germination conditions

Grains of einkorn (*Triticum monococcum* L. ssp. *Monococcum* var. Monlis, TMOm), emmer (*Triticum turgidum* L. ssp. *dicoccum* (Schrank ex Schübler) Thell. var. Zefiro, TDiZ) and durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.) Husnot var. Creso, TDuC) were collected from different locations of Central Italy and were sown in autumn 2014 in small plots of 3 m² without replicates at Mosciano S. Angelo (Central Italy, Province of Teramo, 42.75 °N, 200 m a.s.l., Mediterranean climate). The soil was uniform, silty-clay with 1.2% organic matter. Fertilisation inputs included 60 kg ha⁻¹ of N; no pest and disease control was performed and weeds were controlled by hand. Grains were hand harvested in July 2015, at a grain water content lower than 15% and cleaned from dust and any other contaminants and stored in a dry and cool room until the beginning of the experiment, performed in laboratory.

Germination was carried out to obtain sprouts (growth stage: about 5 cm shoot length) and wheatgrass (growth stage: about 12 cm shoot length) of TMOm, TDiZ and TDuC. Caryopses (200 g for each genotype) were incubated in plastic trays containing filter paper laid over glass balls immersed in 0 (*i.e.* control), 25, 50 and 100 mM NaCl solutions (0_S, 25_S, 50_S and 100_S, respectively) throughout the whole experiment, *i.e.* until either sprout or wheatgrass stage. Additionally two recovery treatments were included consisting of sprouts obtained with 50 and 100 mM NaCl solutions and then transferred in trays containing distilled water until wheatgrass stage (50_R and 100_R, respectively). Each treatment consisted of 4 trays (replicates) arranged on a completely randomised design; replicates were then regrouped two by two for the chemical analysis.

The trays were placed in a growth chamber at 18°C and at light:dark regime of 10:14 hours. Light intensity was set at 200 μmol photons m⁻² s⁻¹, in order to approximate the average light intensity achievable indoor by natural light incoming through a window, with the aim of reproducing the likely environmental con-

ditions for homemade production. Distilled water was periodically added to trays to keep the initial NaCl concentration of each treatment. For 0_S treatments, sprouts were harvested 5 days after the start of incubation (DAS), while wheatgrass was harvested 8 DAS; for all the other treatments, since salinity slowed germination and seedling growth, sprouts and wheatgrass were sampled when they reached the same growth stages as in the control (Table 1). Recovery-treatments (50_R and 100_R) were sampled only at wheatgrass stage, assuming same analysis values as in 50_S and 100_S for sprouts.

For sprouts, the whole plant material was collected, as it is usually in wheat sprouting. By analogy, the whole plant material was taken also for wheatgrass, although only the shoot is normally used to extract juice. Sampled material was stored at -20°C for analytical determinations.

Chemicals

2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), acetic acid, acetonitrile (HPLC grade), ethyl acetate, were purchased from Carlo Erba (Milan, Italy). Sodium carbonate was provided by Panreac (Barcelona, Spain). Folin-Ciocalteu reagent, hydrochloric acid (37% w/v), methanol, sodium hydroxide, gallic acid (GA), caffeic acid (CA), syringic acid (SRA), P-coumaric acid (P-CA), *trans*-ferulic acid (*trans*-FA) and salicylic acid (SA) were purchased from Sigma Aldrich (St. Louis, MO, USA). All standards were prepared as stock solution at 1 mg mL⁻¹ in methanol and stored at -20°C in darkness conditions. Ultra-pure water was obtained from a reverse osmosis system (Technolab, Milan, Italy).

Total polyphenols content

Total polyphenols content (TPC) determination was performed on grains, sprouts and wheatgrass of TMOm, TDiZ and TDuC, using the Folin-Ciocalteu reagent method (Singleton and Rossi, 1965). Grains (10 g) were milled using a laboratory miller and sieved through a 32-mesh screen. Five grams of milled (grains) or freeze-sampled (sprouts and wheatgrass) material for each replicate were treated with MeOH (10 mL) and homogenised with the T-25 Ultra-Turrax (IKA-LAB, Seneco S.r.l., Milano, MI, Italy), then sonicated with Sonis 4 for 1 hour in a cooled water bath. The extracts were centrifuged for 10 minutes at 15,000 g at 4°C and the supernatant was filtered through a Chromafill AO-22/25 polyamide filter. 0.8 mL of Folin-Ciocalteu reagent were added to aliquots of 0.2 mL of extracts; after 5 min, 0.8 mL of a 25% Na₂CO₃ solution was added and then deionised water up to 10 mL final volume. Solutions were maintained at room temperature under dark conditions for 60 min and the total polyphenols content was determined at 765 nm using a Perkin Elmer Lambda Bio20 spectrophotometer (Perkin-Elmer, Waltham, MA, USA). Gallic acid (GA) standard solutions were used to calibrate the method; the contents of total polyphenols in each extract was calculated and expressed as mg of GA equivalent (GAE) per 100 g (dry weight, DW) of sample (mg GAE 100 g⁻¹ DW).

Radical scavenging activity

Radical scavenging activity was measured on TPC extracts, according to the method described by Re *et al.* (1999). ABTS was dissolved in water to a 7 mM concentration; the ABTS radical was obtained from reaction of ABTS stock solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS radical solution was diluted with deionised water to reach an

absorbance of 0.70 ± 0.02 at 734 nm. Thirty μL of differently diluted extracts were added to 2.97 mL of diluted ABTS radical solution. The absorbance at 734 nm was evaluated by a Perkin Elmer Lambda Bio 20 spectrophotometer (Perkin-Elmer, Waltham, MA, USA). For each sample, the percentage of inhibition (I%) after 5 minutes of reaction was plotted as a function of concentration and the Trolox equivalent antioxidant capacity (TEAC) calculated as the ratio of the linear regression coefficient of the sample to that of the Trolox standard (Fluka, Buchs, Switzerland). Results were expressed as μmol of Trolox equivalents (TE) per g (DW) of sample ($\mu\text{mol TE g}^{-1}\text{DW}$).

Extraction of the phenolic fractions (free and bound)

The extraction of free phenolic acids (free-PAs) was achieved on grains, sprouts and wheatgrass of TMoM, TDiZ and TDuC, following the procedure of Vaher *et al.* (2010) with some modifications. One gram of milled (grains) or freeze-sampled (sprouts and wheatgrass) material for each replicate was suspended in 5 mL of MeOH/water/acetic acid (70/29.5/0.5 v/v) and homogenised with the T-25 Ultra-Turrax (IKA-LAB, Seneco S.r.l., Milan, Italy) then sonicated with Sonis 4 for 40 minutes at room temperature. After centrifugation at 4,000 g for 10 min, the supernatant was removed and the extraction was repeated. The combined extracts were evaporated to dryness under a gentle flow of nitrogen and the residues were redissolved in 1 mL of MeOH. The final extract was filtered through 0.45 μm PTFE syringe filters (Phenomenex, Torrance, CA, USA) and stored at -20°C until HPLC analysis.

After extracting free-PAs, the solid residue was hydrolysed according to Kim *et al.* (2006) with minor modification. Briefly, the residue was digested with 10 mL 4M NaOH; the suspension was sonicated for 40 min and treated over night at room temperature. After alkaline hydrolysis the supernatant was adjusted to pH 2 with 6M HCl. The supernatant was extracted three times with ethyl acetate (20 mL each extraction); the mixture was vigorously vortexed for 1 min at room temperature, then centrifuged at 4,000 g for 10 min. The three fractions were pooled, evaporated to dryness under a rotary evaporator in vacuum, redissolved in 2 mL of MeOH and stored at -20°C until HPLC analysis.

All the extractions were performed under dim light to avoid sample degradation by photo-oxidation.

Identification and quantification of phenolic compounds

PAs were analysed using an HPLC system consisting of a Perkin Elmer series 200 LC equipped with a UV/VIS detector and controlled through the software TotalChrom Navigator (Version 6.3.1). The separation of the analytes was carried out with a Kinetex C18 column (250x4.6 mm ID, 5 μm ; Phenomenex, Torrance, CA, USA). The mobile phases were water with 1% (v/v) acetic acid (A) and 100% acetonitrile (B) with a solvent flow rate of 1 mL min^{-1} for a total run time of 60 min. The gradient programme was as follows: from 95% to 84% solvent A in 20 min, from 84% to 30% solvent A in 20 min, from 30% to 95% solvent A in 10 min and then 10 min of post-run for reconditioning. Automatic injections of 20 μL of the standards and sample solutions were carried out; UV chromatograms were recorded at 280 nm (Zhou and Yu, 2004). Identification of PAs was based on their retention times, obtaining suitable chromatographic elution with no interferences or overlapping peaks. Quantitative analysis was based on peak area; the calibration curves, *i.e.* the peak area versus concentration, were linear in the range of the assessed concentrations ($r^2 > 0.992$). The lines of regression, calculated in the range of

5-80 ppm, have been used for quantitative analysis of PAs in the extracts.

Statistical analysis

To test the effect of *Triticum* genotypes (TMoM, TDiZ and TDuC) on TPC, TEAC, free-PAs and bound-PAs in grains, one-way analysis of variance (ANOVA) was performed.

Data on TPC, TEAC, free-PAs and bound-PAs in sprouts and wheatgrass were subjected to two-way ANOVA, with genotypes (TMoM, TDiZ and TDuC) representing the main factor and sprouting conditions (0_S, 25_S, 50_S, 100_S, 50_R and 100_R) the secondary factor.

All the statistical analyses were performed with R software (R Development Core Team, 2013). If the ANOVA detected significant differences, means separation was obtained through the Tukey's honestly significant difference (HSD) test. Prior to ANOVA, data were analysed to test the normality and homoscedasticity assumptions.

Results and discussion

Grains

The results about total polyphenols content in grains of TMoM, TDiZ and TDuC are reported in Figure 1A. TPC values ranged from 30.8 (TDuC) to 40.3 (TMoM) mg GAE $100\text{g}^{-1}\text{DW}$

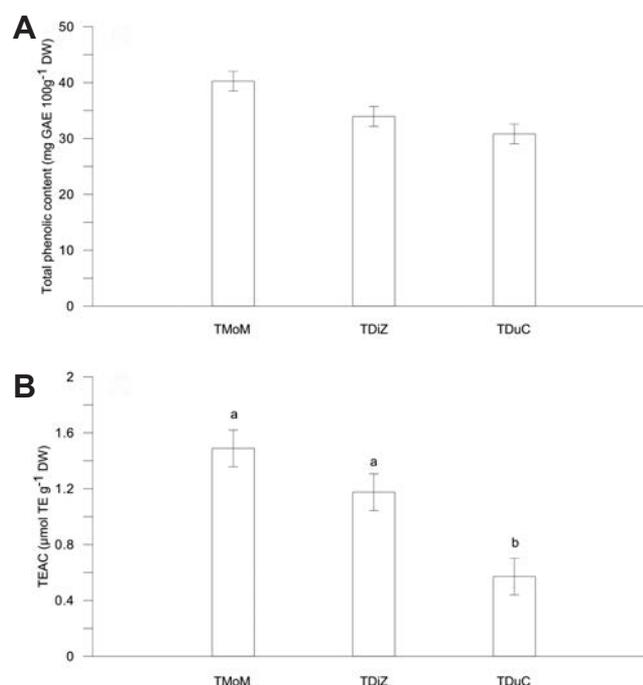


Figure 1. Total phenolic content (mg gallic acid equivalent, GAE, 100g^{-1} dry weight, DW) (A) and antiradical activity (TEAC, μmol Trolox equivalents, TE, g^{-1}DW) (B) in grains of *T. monococcum* cv Monlis (TMoM), *T. dicoccum* cv Zefiro (TDiZ), and *T. durum* cv Creso (TDuC). Data represent means \pm standard errors, $n=2$ independent replicates. Different letters stand for statistically significant differences at $P < 0.05$ (Tukey's honestly significant difference test).

(Figure 1A); significant differences among genotypes were not detected. These data match with the range reported in literature (Shewry and Hey, 2015; Vaher *et al.*, 2010), while differ from Benincasa *et al.* (2015), due to higher TPC in TDuC grains. Probably the particular environmental conditions of 2015 (*i.e.* temperature and rainfall regimes during grain filling) and some abiotic stress factors occurring during kernel development, significantly affected the biosynthesis and accumulation of phenolic compounds (Herbinger *et al.*, 2002).

The effect of genotype was significant in terms of radical scavenging activity, with TMoM and TDuC showing the highest and lowest TEAC values (1.49 and 0.57 $\mu\text{mol TE g}^{-1}$ DW, respectively; Figure 1B), confirming data by Yilmaz *et al.* (2015) although with higher and more variable values probably due to the different extraction's assays.

In order to have a better characterisation of wheat genotypes, providing more detailed information when compared with spectrophotometric methods (such as total polyphenols by Folin-Ciocalteu reagent method), grains extracts were analysed to assess their free and bound phenolic acids fractions. Five phenolic compounds, CA, SRA, P-CA, *trans*-FA and SA were investigated.

In no-sprouted grains, the bound forms accounted for most of the total phenolic acids content (Table 2) as bound-PAs are cross-linked with the mainly structural components such as hydrolysable tannins, lignins, cellulose and proteins (Gawlik-Dzikiet *al.*, 2012). Averaged over genotypes, *trans*-FA showed the highest concentration among total bound-PAs in kernels (Table 2), as previously observed (Benincasa *et al.*, 2015).

Regarding the effect of genotype, the content of total free-PAs in grains of TMoM and TDiZ was more than double compared to that of durum wheat, principally due to higher contents of both P-CA and *trans*-FA (Table 2). An analogous trend was observed for the bound-fraction and the content of total bound-PAs of TMoM and TDiZ which were, on average, over three-fold higher than TDuC (Table 2). Bound *trans*-FA content lowered by about 52 and 56% in TDuC with respect to TMoM and TDiZ, while P-CA was on average 97% lower than the other genotypes, in agreement with Benincasa *et al.* (2015).

Effect of sprouting and salt stress on polyphenols content and radical scavenging activity

Figures 2 and 3 report trends of TPC and TEAC as observed respectively in sprouts and wheatgrass of TMoM, TDiZ and TDuC germinated under normal and salt stress conditions.

Averaged over both genotypes and salt treatments, sprouting enhanced TPC from 6-fold (in sprouts) up to over 20-fold (in wheatgrass) and similar increases were also observed for TEAC values (Figure 4A and B), confirming their significant linear correlation ($r^2=0.98$), as previously observed in some fruits and vegetables (Gorinstein *et al.*, 2007; Kugler *et al.*, 2007). The highest increase was recorded for TDuC, the genotype with the lowest

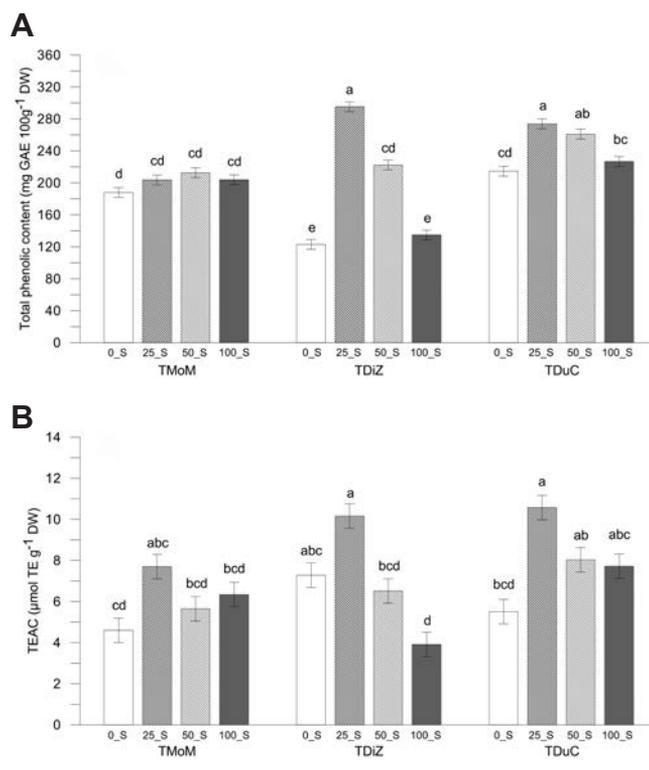


Figure 2. Total phenolic content (mg gallic acid equivalent, GAE, 100 g^{-1} dry weight, DW) (A) and antiradical activity (TEAC, $\mu\text{mol Trolox equivalents, TE g}^{-1}$ DW) (B) in sprouts of *T. monococcum* cv Monlis (TMoM), *T. dicoccum* cv Zefiro (TDiZ), and *T. durum* cv Creso (TDuC), obtained under 0 (*i.e.* control), 25, 50 and 100 mM NaCl solutions (0_S, 25_S, 50_S and 100_S, respectively). Data represent means \pm standard errors, $n=2$ independent replicates. Different letters stand for statistically significant differences at $P < 0.05$ (Tukey's honestly significant difference test).

Table 1. Sampling details of sprouts and wheatgrass of einkorn (*Triticum monococcum* L. ssp. *monococcum* var Monlis), emmer (*Triticum turgidum* L. ssp. *dicoccum* (Schrank ex Schübler) Thell. var Zefiro) and durum wheat (*Triticum turgidum* L. ssp. *durum* (Desf.) Husnot var Creso) during germination.

Treatments	TMoM		TDiZ		TDuC	
	Sprouts ^o DAS	Wheatgrass [#] DAS	Sprouts DAS	Wheatgrass DAS	Sprouts DAS	Wheatgrass DAS
0_S	5	8	5	8	5	8
25_S	5	8	5	8	5	8
50_S	6	9	6	11	6	10
100_S	7	10	8	13	7	11
50_R	-	9	-	9	-	9
100_R	-	9	-	9	-	9

TMoM, *Triticum monococcum* cv Monlis; TDiZ, *Triticum dicoccum* cv Zefiro; TDuC, *Triticum durum* cv Creso; DAS, days after the start of incubation. ^oGrowth stage: about 5 cm shoot length; [#]growth stage: about 12 cm shoot length. Grains were subjected to 0 (*i.e.* control), 25, 50 and 100 mM NaCl solutions (0_S, 25_S, 50_S and 100_S, respectively) throughout the whole experiment, or to 50 and 100 mM NaCl solutions until sprout stage and then to 0 mM until wheatgrass stage (recovery-treatments: 50_R and 100_R, respectively).

TPC and TEAC values in grains (TPC: up to 7-fold in sprouts, up to 27-fold in wheatgrass; TEAC: up to 13-fold in sprouts, up to 38-fold in wheatgrass) (Figure 4A).

The observed increase in total phenolic content and antiradical activity confirmed evidence obtained in cereals (Benincasa *et al.*, 2015; Donkor *et al.*, 2012) as well as in several other species belonging to *Leguminosae* and *Brassicaceae* families (Cevallos-

Casals and Cisneros-Zevallos, 2010; Dueñas *et al.*, 2009). Germination leads to significant changes in phenolic composition, mainly due to the activation of endogenous hydrolytic enzymes, which in turn affect the antiradical activity of sprouts (Dueñas *et al.*, 2009).

Modifications of sprouting conditions (*i.e.* salt-stress during germination) aimed at overproducing antioxidants *inter alia* phenolic compounds, usually involve induction of the natural mechanisms of plant resistance. In our study, the interaction *Genotype x NaCl* was significant in terms of total polyphenols contents in sprouts and wheatgrass of TMoM, TDiZ and TDuC ($P < 0.05$; Figures 2A and 3A, respectively). A similar genotype-specific response to different magnitudes of salt stress was also observed by Danai-Tambhale *et al.* (2011) in 21-day old seedling of two indica rice cultivars, indicating the importance of genetic variability on salt tolerance (Yuan *et al.*, 2010). In general, sprouts of TMoM did not show differences on TPC values when exposed to different salinity conditions; moreover, at wheatgrass growth stage TPC values were significantly higher only at 100 mM NaCl solution (1082 mg GAE 100 g⁻¹ DW; Figure 3A). This trend is in

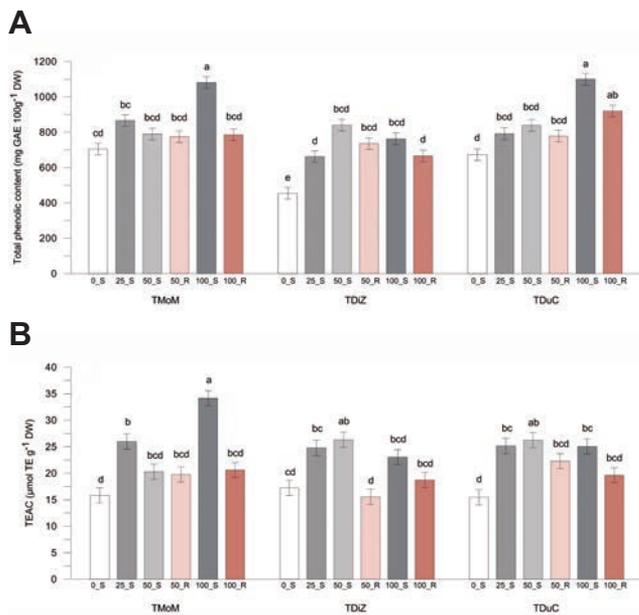


Figure 3. Total polyphenols content (mg gallic acid equivalent, GAE, 100 g⁻¹ dry weight, DW) (A) and antiradical activity (TEAC, μmol Trolox equivalents, TE, g⁻¹ DW) (B) in wheatgrass of *T. monococcum* cv Monlis (TMoM), *T. dicoccum* cv Zefiro (TDiZ), and *T. durum* cv Creso (TDuC), obtained under 0 (*i.e.* control), 25, 50 and 100 mM NaCl solutions (0_S, 25_S, 50_S and 100_S, respectively) throughout the whole experiment (*i.e.* until wheatgrass stage), or under 50 and 100 mM NaCl solutions until sprout stage and then transferred to 0 mM until wheatgrass stage (recovery-treatments: 50_R and 100_R, respectively). Data represent means ± standard errors, n=2 independent replicates. Different letters stand for statistically significant differences at $P < 0.05$ (Tukey's honestly significant difference test).

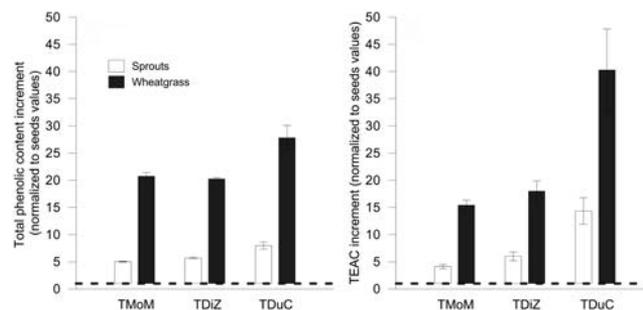


Figure 4. Increment normalised to grains values (dashed line) of total polyphenols content (mg gallic acid equivalent 100 g⁻¹ dry weight, DW) (A) and antiradical activity (TEAC, μmol Trolox equivalents g⁻¹ DW) (B) as observed in *T. monococcum* cv Monlis (TMoM), *T. dicoccum* cv Zefiro (TDiZ), and *T. durum* cv Creso (TDuC) during germination, averaged over salt treatments. See Table 1 and text for further explaining. Data represent means ± standard errors, n=2 independent replicates.

Table 2. Free and bound phenolic acids content (both μg g⁻¹ dry weight) in grains of *T. monococcum* cv Monlis, *T. dicoccum* cv Zefiro, and *T. durum* cv Creso.

Genotypes		PAs (μg g ⁻¹ DW)					
		CA	SRA	P-CA	trans-FA	SA	Total ^o
Free-PAs	TMoM	nd	nd	1.74 ^b	1.43 ^{ab}	nd	3.17 ^a
	TDiZ	nd	nd	2.09 ^a	1.61 ^a	nd	3.70 ^a
	TDuC	nd	nd	0.40 ^c	1.25 ^b	nd	1.65 ^b
	Significance			**	*		**
	SED			0.06	0.07		0.11
Bound-PAs	TMoM	17.96	3.99 ^b	541.35 ^a	729.80 ^a	47.07	1340.17 ^a
	TDiZ	14.63	5.14 ^a	529.56 ^a	673.51 ^a	nd	1222.85 ^b
	TDuC	13.00	nd	13.90 ^b	316.99 ^b	nd	343.89 ^c
	Significance	ns	**	**	**	-	**
	SED	11.08	0.41	8.13	29.62	-	26.95

PAs, phenolic acids; DW, dry weight; CA, caffeic acid; SRA, syringic acid; P-CA, P-coumaric acid; trans-FA, trans-ferulic acid; SA, salicylic acid; TMoM, *Triticum monococcum* cv Monlis; TDiZ, *Triticum dicoccum* cv Zefiro; TDuC, *Triticum durum* cv Creso; SED, standard error of differences between means; nd, not detectable. * $P < 0.05$; ** $P < 0.01$; ns, not-significant. ^oSum of all detected PAs. Degrees of freedom: Genotype 2; residual 3. *^oDifferent letters stand for statistically significant differences at $P < 0.05$ (Tukey's honestly significant difference).

agreement with the studies of Guo *et al.* (2014) and Yuan *et al.* (2010) who found a significant increase in TPC in radish and broccoli sprouts, but only at the highest stressful condition. Conversely, the effect of salt stress significantly enhanced TPC of both TDiZ and TDuC genotypes already during the very early stages (Figure 2A) and the highest values were reached by 25_S treatments (295 and 274 mg GAE 100 g⁻¹ DW, in TDiZ and TDuC, respectively). For these genotypes, at the highest salt concentration (100_S treatment) the TPC levels were similar to the unstressed control (Figure 2A). The prolonged exposure to salinity, as observed at wheatgrass stage, enhanced TPC values in TDuC up to 100_S treatment (Figure 3A); no differences between salt-treatments were observed in TDiZ (Figure 3A). Regardless of genotypes, recovery-treatments lead to a reduction on TPC, especially for those previously subjected to the highest stressful condition; however, it has to be pointed out that TPC values in 50_R and 100_R treatments were generally higher than in the unstressed control (Figure 3A).

The trend of the radical scavenging activity, as a function of salt stress levels, resulted often unclear and not always in agreement with TPC data, strictly depending on growth stage and genotypes; for this reason it does not seem meaningful and worth speculating on. Anyway, averaged over genotypes, we found a signifi-

cant linear correlation between TPC and TEAC values at sprout stage ($r^2=0.81$), which was reduced at wheatgrass stage ($r^2=0.72$).

Effect of sprouting and salt stress on free- and bound-phenolic acids

Both free and bound fractions content of the detected PAs in sprouts and wheatgrass of TMoM, TDiZ and TDuC are presented in Tables 3 and 4, respectively.

The total free-PAs content ranged from 10.5 to 27.2 µg g⁻¹ DW in sprouts (Table 3) and from 18.4 to 85.8 µg g⁻¹ DW in wheatgrass (Table 4). During germination the total free-PAs content increased significantly by over 6-fold at sprout stage (averaged over genotypes and salt treatments) (Figure 5A). Then, a further increase from sprout to wheatgrass stage was observed and the free-PAs content averagely increased by about 17-fold with respect to grains (Figure 5A), confirming results on a FW basis reported by Benincasa *et al.* (2015). Genotypes gave different response to germination process with the highest free-PAs increase observed in TDuC.

Total bound PAs ranged from 334.2 to 1898.8 µg g⁻¹ DW in sprouts (Table 3) and from 208.0 to 7769.8 µg g⁻¹ DW in wheat-

Table 3. Free and bound phenolic acids content (both µg g⁻¹ dry weight) in sprouts of *T. monococcum* cv Monlis, *T. dicoccum* cv Zefiro, and *T. durum* cv Creso, obtained under 0 (*i.e.* control), 25, 50 and 100 mM NaCl solutions.

Genotypes			CA	SRA	PAs (µg g ⁻¹ DW)		SA	Total°
					P-CA	trans-FA		
Free-PAs	TMoM	0_S	nd	nd	5.1 ^{cd}	12.8 ^{bc}	nd	17.9 ^{cd}
		25_S	nd	nd	5.3 ^{cd}	21.9 ^a	nd	27.2 ^a
		50_S	nd	nd	6.2 ^{bcd}	14.0 ^{bc}	nd	20.2 ^{bc}
		100_S	nd	nd	4.1 ^d	13.6 ^{bc}	nd	17.7 ^{cd}
	TDiZ	0_S	nd	nd	7.8 ^{abc}	9.8 ^{cde}	nd	17.6 ^{cd}
		25_S	nd	nd	6.9 ^{bcd}	17.7 ^{ab}	nd	24.6 ^{ab}
		50_S	nd	nd	8.7 ^{ab}	13.8 ^{bc}	nd	22.5 ^{abc}
		100_S	nd	nd	5.6 ^{bcd}	7.2 ^{de}	nd	12.8 ^{de}
	TDuC	0_S	nd	nd	5.4 ^{cd}	5.1 ^e	nd	10.5 ^e
		25_S	nd	nd	7.3 ^{bc}	13.0 ^{bc}	nd	20.3 ^{bc}
		50_S	nd	nd	10.7 ^a	12.9 ^{bc}	nd	23.6 ^{ab}
		100_S	nd	nd	10.9 ^a	11.8 ^{cd}	nd	22.6 ^{abc}
		Significance	Genotype		**	**		ns
			NaCl		**	**		**
		Genotype x NaCl		**	**		**	
	SED		0.81	1.31		1.42		
Bound-PAs	TMoM	0_S	nd	nd	410.8 ^{de}	410.5 ^{de}	21.1	842.5 ^{ef}
		25_S	nd	nd	641.4 ^b	757.4 ^b	27.6	1426.5 ^{bc}
		50_S	nd	nd	881.0 ^a	979.5 ^a	38.1	1898.8 ^a
		100_S	nd	nd	356.7 ^{de}	366.0 ^{def}	15.9	738.8 ^f
	TDiZ	0_S	nd	nd	477.7 ^{cd}	562.4 ^{bcd}	23.7	1063.8 ^d
		25_S	nd	nd	583.9 ^{bc}	744.2 ^b	35.2	1363.4 ^c
		50_S	nd	nd	676.0 ^b	749.1 ^b	68.8	1494.0 ^{bc}
		100_S	nd	nd	141.1 ^f	161.7 ^f	31.4	334.2 ^g
	TDuC	0_S	63.0	nd	468.4 ^{cd}	1056.9 ^a	nd	1588.4 ^b
		25_S	32.6	nd	291.8 ^e	654.7 ^{bc}	nd	979.1 ^{de}
		50_S	33.9	nd	104.4 ^f	522.3 ^{cd}	nd	660.7 ^f
		100_S	32.9	nd	74.8 ^f	229.6 ^{ef}	nd	337.5 ^g
		Significance	Genotype		**	*		**
			NaCl		**	**		**
		Genotype x NaCl		**	**		**	
	SED			33.44	54.37		48.09	

PA, phenolic acid; DW, dry weight; CA, caffeic acid; SRA, syringic acid; P-CA, P-coumaric acid; trans-FA, *trans*-ferulic acid; SA, salicylic acid; TMoM, *T. monococcum* cv Monlis; TDiZ, *T. dicoccum* cv Zefiro; TDuC, *T. durum* cv Creso; SED, standard error of differences between means; nd, not detectable. *P<0.05; **P<0.01; ns, not significant. °Sum of all detected PAs. Degrees of freedom: Genotype 2; NaCl 3; Genotype x NaCl 6; residual 12. Data are the means for n=2 independent replicates. **Different letters stand for statistically significant differences at P<0.05 (Tukey's honestly significant difference).

grass (Table 4), contributing heavily to the total detected PAs (free-PAs plus bound-PAs). Also in this case, bound-PAs content tended to increase over germination although not at the same extent as the free- ones, from 0.5-fold to about 2.5-fold in sprouts and wheatgrass, respectively (averaged over genotypes and salt treatments) (Figure 5B).

The effect of genotype was particularly clear: in TMoM and TDiZ the content of total-bound PAs were lower at sprout stage with respect to grains (Figure 5B). An early reduction in the TPC of bound phenolic extracts was also observed by Hung *et al.* (2012) in waxy wheat germinated grains, and it was attributed to a first lost hydrolysis of polyphenolic compounds bound to cell walls (Yang *et al.*, 2001). Moreover, during the first stages of germination the decline of bound phenolic concentration coincided with a rise of the free-fraction, indicating a shift between two forms (Xu *et al.*, 2009). However, it is improbable to compare our results with the literature because of the different germination time (5-days vs 2-days old sprouts).

HPLC analyses showed that the qualitative compositions of the free- and bound- phenolic fractions of TMoM, TDiZ and TDuC during germination did not substantially change (Tables 2, 3 and 4). In sprouts, *trans*-FA contributed to 63% and 57% of the total free-PAs and bound-PAs, respectively (Table 3). The *trans*-FA was also the most represented PA in wheatgrass (65% of the total free-PAs and 64% of the total bound-PAs; Table 4) (Hung *et al.*, 2011).

Both free- and bound-PAs fractions in sprouts extracts were significantly affected by *Genotype x NaCl* interaction ($P < 0.05$; Table 3). In TMoM sprouts, salt-stress induced significant differences in terms of total free-PAs only at 25_S, in part confirming TPC data. However, salinity significantly enhanced total bound-PAs (highest value at 50_S; Table 3). Interestingly, the increase in the bound-fraction was related to a significant induction of both P-CA and *trans*-FA. Cell-wall-bound FA has been demonstrated to cope with drought tolerance in triticale seedlings, acting as regulator of cell growth by ester and decreasing cell-wall extensibility and sprout elongation at the cost of osmotic adjustment (Hura *et al.*, 2009). Conversely, salt-stress raised up total free-PAs till 50_S in sprouts of TDuC, whilst significantly lowered total bound-PAs (Table 3). The negative variation of total bound-PAs mainly depended on the variation of both P-CA and *trans*-FA. In general, salt-stress treatment induced an increase in the concentration of both free- and bound-phenolic fractions in sprouts extracts of TDiZ, although the differences were not always significant (Table 3). The contribution to total bound-PAs increase is also attributable to SA, which showed the highest values in 50_S treatment (Table 3) and which could act as a signaling molecule inducing salt tolerance in plants (Jayakannan *et al.*, 2015).

With respect to wheatgrass extracts, the content of total free-PAs in response to NaCl treatments showed similar trends to sprouts ones, although differences on specific PAs were observed (Table 4). Regarding the bound-fraction, total detected bound-PAs in TMoM showed an upward trend until 50_S treatment; further NaCl level significantly constrained total bound-PAs values (Table 4). The same trend was observed for P-CA and *trans*-FA. However, the recovery-treatment 100_R allowed increasing total bound-PAs with respect to 100_S, thanks to the contribution of *trans*-FA and SA in their bound forms (Table 4). Interesting, SA became detectable in its bound form also in 50_R and 100_R treatments (Table 4). We hypothesised that TMoM, given optimal growing conditions, is promptly adaptable to stressful levels during germination with SA being involved in this process. On the other hand, in TDuC P-CA, *trans*-FA and, consequently, total-PAs in their

bound forms significantly decreased in response to prolonged salt-stress treatments (Table 4). For TDiZ, despite an increase in total bound-PAs until 50_S, any significant difference was detected (Table 4). This could be attributable also to the reduction of bound SRA that became undetectable under severe stressful conditions, even if the optimal growing conditions were restored (see 50_R and 100_R treatments; Table 4). Interestingly, TDiZ represented the genotype characterised by higher SA content with respect to TMoM and TDuC (Table 4).

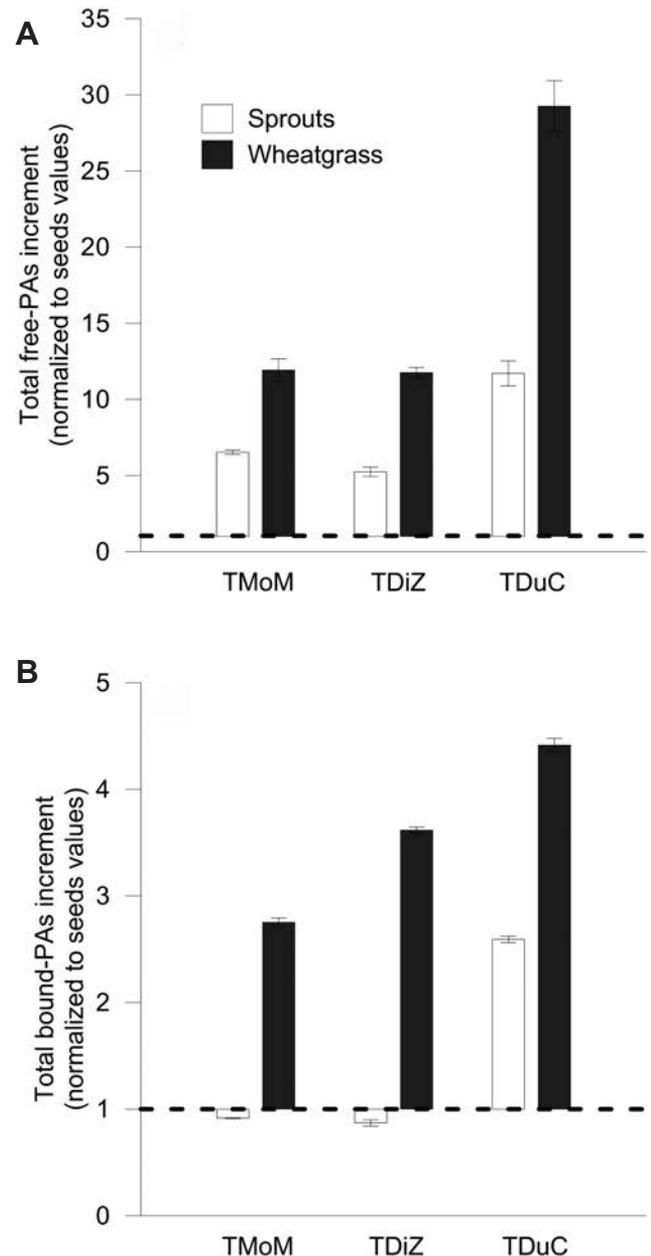


Figure 5. Increment normalised to grains values (dashed line) of total free-phenolic acids (PAs) ($\mu\text{g g}^{-1}$ dry weight, DW) (A) and total bound-PAs ($\mu\text{g g}^{-1}$ DW) (B) in *T. monococcum* cv Monlis (TMoM), *T. dicoccum* cv Zefiro (TDiZ), and *T. durum* cv Creso (TDuC) during germination, averaged over salt treatments. See Table 1 and text for further explanation. Data represent means \pm standard errors, $n=2$ independent replicates.

Conclusions

Sprouting significantly enhanced TPC and detected bound and free-PAs, regardless of genotype. Response to salt stress depended on genotype sensitivity, growth stage and salt concentration. In general, moderate salinity levels (not exceeding 50 mM NaCl solution) induced accumulation of phenolic acids in both sprouts and wheatgrass of *Triticum* genotypes with a pronounced variability. In particular, in TMoM the bound-PAs fraction of both

sprout and wheatgrass extracts increased until the dose of 50 mM NaCl, probably thanks to the induction of P-CA and *trans*-FA. Conversely, in TDuC, NaCl determined an increase of PAs in their free form (as confirmed by TPC data) and a reduction of the bound ones. In TDiZ salinity generally enhanced the TPC, TEAC, free-PAs and bound-PAs content, although the responses were not always significant.

However, to better understand the species-specific response to salt, further researches on a wider number of both genotypes and growing environments, are needed.

Table 4. Free and bound phenolic acids content (both $\mu\text{g g}^{-1}$ dry weight) as observed in wheatgrass of *T. monococcum* cv Monlis, *T. dicoccum* cv Zefiro, and *T. durum* cv Creso, obtained with 0 (*i.e.* control), 25, 50 and 100 mM NaCl solutions throughout the whole experiment (*i.e.* until wheatgrass stage), or with 50 and 100 mM NaCl solutions until sprout stage and then transferred to 0 mM NaCl until wheatgrass stage.

Genotypes			PAs ($\mu\text{g g}^{-1}$ DW)					
			CA	SRA	P-CA	<i>trans</i> -FA	SA	Total ^a
Free-PAs	TMoM	0_S	nd	nd	6.0 ^{de}	36.3 ^{bcd}	nd	42.3 ^{bcd}
		25_S	nd	nd	5.2 ^{de}	26.1 ^{def}	nd	31.3 ^d
		50_S	nd	nd	5.2 ^{de}	32.1 ^{bcde}	nd	37.3 ^{cd}
		50_R	nd	2.3	10.1 ^{bcde}	16.6 ^{ef}	nd	28.9 ^d
		100_S	nd	nd	2.7 ^e	19.0 ^{ef}	nd	21.7 ^d
	TDiZ	100_R	8.7	9.4	14.9 ^{bc}	32.3 ^{bcde}	nd	65.3 ^{ab}
		0_S	nd	nd	15.9 ^b	19.1 ^{ef}	nd	35.0 ^d
		25_S	nd	nd	11.4 ^{bcd}	17.0 ^{ef}	nd	28.4 ^d
		50_S	nd	nd	14.9 ^{bc}	58.1 ^a	nd	73.0 ^a
		50_R	nd	nd	16.8 ^b	45.0 ^{abc}	nd	61.8 ^{abc}
	TDuC	100_S	nd	nd	7.6 ^{cde}	18.9 ^{ef}	nd	26.5 ^d
		100_R	nd	nd	11.6 ^{bcd}	24.7 ^{def}	nd	36.3 ^{cd}
		0_S	nd	nd	28.7 ^a	27.0 ^{def}	22.9	78.6 ^a
		25_S	nd	nd	13.1 ^{bcd}	29.0 ^{cdef}	nd	42.1 ^{bcd}
		50_S	nd	nd	33.0 ^a	46.8 ^{ab}	5.96	85.8 ^a
		50_R	nd	nd	5.8 ^{de}	12.6 ^f	nd	18.4 ^d
		100_S	nd	nd	26.1 ^a	13.5 ^f	nd	39.6 ^{bcd}
		100_R	nd	nd	11.3 ^{bcd}	14.5 ^f	nd	25.8 ^d
		Significance		Genotype	**	**		**
				NaCl	**	**		**
		Genotype x NaCl	**	**		**		
	SED		1.99	4.08		6.44		
Bound-PAs	TMoM	0_S	nd	nd	1688.8 ^c	2250.7 ^d	nd	3939.4 ^{cd}
		25_S	nd	nd	1971.5 ^b	2353.5 ^{cd}	nd	4325.0 ^{bcd}
		50_S	nd	nd	2150.7 ^b	2711.8 ^c	nd	4862.5 ^b
		50_R	nd	nd	1525.0 ^{bc}	2702.7 ^c	177.2	4404.9 ^{bc}
		100_S	nd	nd	579.9 ^{def}	519.0 ^{gh}	nd	1098.9 ^f
	TDiZ	100_R	nd	nd	929.5 ^{cde}	2477.6 ^{cd}	95.6	3502.7 ^d
		0_S	nd	26.9	3416.8 ^a	3726.6 ^a	248.7	7419.1 ^a
		25_S	nd	43.0	3521.4 ^a	3792.6 ^a	228.1	7585.1 ^a
		50_S	nd	22.9	3779.5 ^a	3761.5 ^a	205.9	7769.8 ^a
		50_R	nd	nd	288.4 ^{def}	1572.4 ^e	155.6	2016.4 ^e
	TDuC	100_S	nd	nd	427.4 ^{def}	488.2 ^{gh}	52.3	967.8 ^{fg}
		100_R	nd	nd	159.4 ^{ef}	553.7 ^g	73.3	786.3 ^{fg}
		0_S	nd	19.0	1045.9 ^{cd}	3138.6 ^b	nd	4203.5 ^{bcd}
		25_S	nd	2.1	308.8 ^{def}	956.0 ^f	nd	1266.9 ^{ef}
		50_S	nd	nd	246.4 ^{ef}	1137.7 ^f	nd	1384.1 ^{ef}
		50_R	nd	nd	177.8 ^{ef}	1213.2 ^{ef}	nd	1391.1 ^{ef}
		100_S	nd	nd	162.3 ^{ef}	488.9 ^{gh}	nd	651.3 ^{fg}
		100_R	nd	nd	63.9 ^f	144.1 ^h	nd	208.0 ^g
		Significance		Genotype	**	**		**
				NaCl	**	**		**
		Genotype x NaCl	**	**		**		
	SED		198.74	96.88		217.90		

PA, phenolic acid; DW, dry weight; CA, caffeic acid; SRA, syringic acid; P-CA, P-coumaric acid; *trans*-FA, *trans*-ferulic acid; SA, salicylic acid; TMoM, *Triticum monococcum* cv Monlis; TDiZ, *Triticum dicoccum* cv Zefiro; TDuC, *Triticum durum* cv Creso; SED, standard error of differences between means; nd, not detectable. **P<0.01. ^aSum of all detected PAs. Degrees of freedom: Genotype2; NaCl 5; Genotype x NaCl 10; residual18. Data are the means for n=2 independent replicates. ^{a-h}Different letters stand for statistically significant differences at P<0.05 (Tukey's honestly significant difference).

These results indicate that salt stress could be efficiently modulated in order to improve the nutritional value of cereal sprouts and wheatgrass, through the accumulation of their bioactive compounds. This could provide the basis for further *in vivo* investigations to understand the impact of the enriched-quality sprouts-based products on human health.

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