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Foliar application of nano-Zn and mycorrhizal inoculation enhanced Zn in grain and yield of two barley (*Hordeum vulgare*) cultivars under field conditions

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Abstract

Zinc (Zn) deficiency is a global micronutrient problem in agricultural systems. The main target of this experiment was to investigate the effectiveness of foliar application of Zn under field conditions. Grain yield and Zn concentration in seed were assessed with three replicate plots per treatment in a factorial (2 x 3 x 2) experiment for two barley cultivars (Yusuf and Julgeh), three foliar ZnO applications (nano, ordinary and nano+ordinary ZnO) and two commercial inocula of arbuscular mycorrhizal (AM) fungi (*F. mosseae* and *R. irregularis*). Among all Zn foliar applications, Zn applied in both nano and nano+ordinary forms were labile and resulted in the highest Zn concentration in grain of both barley cultivars. Cultivar Julgeh had higher grain Zn concentrations than did cultivar Yusuf in the same treatments. Nano ZnO was more effective than the ordinary form of ZnO and had the highest potential to improve physiological traits, plant growth and yield parameters in both cultivars. There was also a positive impact of the nano form of ZnO on phytase activity and carbonic anhydrase concentration in both barley cultivars. Inoculation with commercial inocula of AM fungi also enhanced grain Zn concentration, with Julgeh more responsive to inoculation with *F. mosseae*, and Yusuf more responsive to inoculation with *R. irregularis*. Generally, the combined application of Zn and inoculation with AM fungi improved physiological traits, grain yield and Zn availability to these two barley cultivars grown under field conditions. Accordingly, the nano form of Zn positively enhanced shoot morphological parameters, physiological parameters and grain Zn concentration. Application of the nano form ZnO in combination with inoculation with AM fungi had the most beneficial effects on grain Zn concentration, so this combined practice may have potential to reduce the requirement for application of synthetic Zn chemical fertilizers.

Keywords: Barley, diverse Zn spraying, AMF symbiosis, yield related traits.
Abbreviations: CH.T_ Chlorophyll total; SS_ Soluble sugar; CA_ Carbonic anhydrase; GPH_ Grain phytase activity; GZN_ Grain zinc; GN_ Grain number; FLA_ Flag leaf Area; TKW_ Thousand Kernel weight; GY_ Grain yield; BY_ Biological yield; HI_ Harvest Index; SY_ Straw Yield.

Introduction

Micronutrients have a pivotal role in global agriculture. Zinc (Zn) uptake by roots is reduced when soil moisture and organic matter are reduced (Rengel, 2015). Deficiency in micronutrients in calcareous soil of arid and semi-arid areas is a principle factor that decreases seed vigor, viability and germination rate, as well as crop growth and yield (Yilmaz et al., 1998; Khademi et al., 2006).

Interactions between phosphorus (P) and Zn in the rhizosphere, especially due to excessive use of P fertilizer, can result in an imbalance between these essential elements in plant tissue, leading to Zn deficiency in shoots and grain (Khan et al., 2014). According to Zhang et al. (2012), P applications decreased grain Zn concentration by 17–56% in grain of wheat (*Triticum aestivum* L.) cultivars in a field experiment. They overcame this imbalance between P and Zn using foliar application of Zn. Greater Zn absorption following foliar application can elevate photosynthetic activities as well as improve transport of Zn into storage organs leading to yield enhancement (Sundaram and Stalin, 2016). The imbalance between P and Zn is not the only problem (Velu et al., 2014), as P, a major component of phytate, can negatively influence the digestibility and availability of Zn stored in edible parts of plants and grain (Lott et al., 2000). Phytate can reduce the bioavailability of P, calcium (Ca), Zn and other metabolically important minerals in animal and human diets (Harland and Oberleas, 2010). Zn availability on the other hand influences the amount of phytases in cereal grains and legume seeds cause to hydrolyse phytate (Greiner and Konietzny, 2006; Ram et al., 2010) thereby increasing the nutritional value of grain. Foliar application of $ZnSO_4$ was shown to be effective in increasing Zn in wheat grain (Velu et al., 2014). This was also observed by Erdal et al. (2002) who reported that the amount of phytase in some wheat cultivars increased following addition of Zn fertilizer to soil. However, the efficacy of foliar application of Zn has not been investigated for barley.

Foliar application of Zn is an effective pathway for increasing plant growth in Zn deficient soil and reduces the potential for water pollution from an excess of soil-applied nutrients with potential for more economical and sustainable agriculture (Hamayun et al., 2011). Zn is an essential component of carbonic anhydrase which is classified as a metallo enzyme in terms of the presence of an essential atom of Zn as a cofactor (So et al., 2004). Under Zn deficiency in rice, the expression of carbonic anhydrase mRNA was reduced by about 13% due to a reduction in the amount of the enzyme (Sasaki et al., 1998).

Foliar application of nutrients in the form of nanomaterials has been shown to overcome deficiencies for plant growth (Giraldo et al., 2014). There is evidence that benefits of foliar application of the nano form of microelements such as Fe and Zn is due to the smaller particle size (diameter and weight) compared to ordinary forms, leading to reduced leaching, increased solubility, and rapid absorption by plants in comparison to traditional fertilizers (Fedorenko et al., 2015). Small-diameter nanoparticles of less than 100 nm alter physical and chemical properties of fertilizers (Monica and Cremonini, 2009) compared to usual forms of nutrients, including ZnO (McBeath and McLaughlin, 2014). Therefore, novel nanofertilizers, especially for elements with low bioavailability in the rhizosphere, can be more effective than traditional fertilizers (A El-Kereti et al., 2013). Although impacts of foliar- and soil-applied Zn on physiological, morphological parameters and plant yield have been investigated (Torabian et al., 2016; Sundaram and Stalin, 2016; Cakmak et al., 2010), few studies have compared the efficiency of ordinary and nano forms of ZnO applied to soil and foliage.

In addition to foliar application of Zn, improving the effectiveness of the symbiosis between arbuscular mycorrhizal (AM) fungi and host plants is another strategy which has potential to assist absorption of Zn associated with hyphal transport, biochemical alterations in the rhizosphere and plant physiological changes (Subramanian et al., 2011). Advantages of AM fungi and their potential role in improving Zn nutrition in numerous crop species have been investigated, but barley has less commonly been considered (Watts-Williams and Cavagnaro, 2018). Subramanian et al. (2011) highlighted the potential of AM fungi in solubilization of residual Zn (fixed form) into exchangeable or organically bound Zn (soluble Zn) to improve its availability. In this case, significant increases in grain yield, harvest index and concentrations of P, N, Zn in shoots of wheat were observed following inoculation with AM fungi in the field (Pellegrino et al., 2015).

Based on the need for more field studies of novel fertilizers and responses to inoculation with AM fungi, the experiment presented here addresses this in terms of alleviation of Zn deficiency in barley, particularly in Iran. This field study investigated effects of the form of foliar Zn spray, with or without use of commercial inocula of AM fungi, in eliminating Zn deficiency in grain for two barley cultivars. The aims were (i) to determine the suitability of various forms of Zn foliar sprays on biochemical traits as well as yield parameters of barley, (ii) to compare the efficiency of using two commercial inocula of AM fungi in improving yield and grain Zn concentration of two barley cultivars, and (iii) to compare the efficiency of mycorrhizal inoculation in combination with foliar application of Zn to improve the Zn concentration in barley grain.

Results

Physiological traits

Chlorophyll content in barley leaves

Chlorophyll content differed between cultivars upon Zn treatments and mycorrhizal inoculation (*P*<0.01; Supplementary Table 2). However, there was no interaction between cultivar and Zn application. Chlorophyll content (mg g^{-1}fw) was enhanced by Zn spray and mycorrhizal inoculation in both cultivars (Supplementary Table 2). The maximum chlorophyll content (8.35 mg g^{-1}fw) occurred in Julgeh inoculated with *F. mosseae* when sprayed with mixed nano+ordinary ZnO. The lowest chlorophyll content (2.19 mg g^{-1}fw) was recorded for Yusuf in the control treatment (Fig. 1a).

Soluble sugar concentration in leaves of barley

Soluble sugar concentration in leaves differed between cultivars, and with Zn application and mycorrhizal inoculation, and there were interactions between them (*P*<0.01; Supplementary Table 2). The highest soluble sugar concentration was observed for the combination of Zn foliar application and mycorrhizal inoculation. The maximum soluble sugar concentration in Julgeh was obtained following spraying with nano+ordinary ZnO and inoculation with *F. mosseae*. The highest sugar concentration in Yusuf was observed for plants inoculated with *R. irregularis* with the nano+ordinary ZnO treatment but it was 45% less than that recorded for Julgeh (Fig. 1b). Positive correlations between soluble sugar and the majority of yield parameters were highly significant (*P*<0.01; Table 2).

Carbonic anhydrase concentration in leaves of barley

Foliar Zn application and mycorrhizal inoculation both significantly influenced carbonic anhydrase concentration for the barley cultivars (*P*<0.01; Supplementary Table 2). There were interactions between cultivars and mycorrhizal inoculation, cultivars and Zn treatments and Zn treatments and mycorrhizal inoculation (*P*<0.01; Supplementary Table 2). The highest level of carbonic anhydrase was observed in Julgeh inoculated with *F. mosseae* when sprayed with nano+ordinary ZnO and the lowest level of carbonic anhydrase occurred for Yusuf in the control (Fig. 1c). Application of Zn via foliar application or mycorrhizal inoculation enhanced Zn supply to both barley cultivars with a corresponding increase in carbonic anhydrase. Correlations between carbonic anhydrase and the majority of traits were positive ($P<0.01$; Table 2).

Phytase activity in barley grains

Grain phytase activity showed a large genotypic variation, and the greatest phytase activity occurred for Julgeh inoculated with *F. mosseae* and sprayed with nano+ordinary ZnO and the lowest phytase activity was observed for Yusuf in the control (Fig. 2a)

Zinc concentration in barley grains

Foliar Zn application to barley leaves increased grain Zn concentration. All three factors had a significant effect on grain Zn concentration and there was a significant interaction between them ($P<0.01$; Supplementary Table 2). The highest Zn concentration in barley grain (62 mg Zn kg^{-1}) occurred for Julgeh inoculated with *F. mosseae* when sprayed with a mixture of nano+ordinary ZnO compared to untreated control (20 mg Zn kg^{-1}) (Fig 2b). Although the concentration of Zn in Yusuf grain was enhanced by inoculation with mycorrhizal fungi and application of foliar Zn, this trend was less marked in Julgeh (Fig.2b). The nano form of ZnO had greater effects on all physiological traits and yield parameters measured compared with the ordinary form of ZnO. Although application of all forms of ZnO increased grain Zn concentration, nano ZnO was more effective.

Shoot and grain traits of barley cultivars for Zn and mycorrhizal treatments

Foliar Zn applications and mycorrhizal inoculation both significantly increased number of grains per plant, flag leaf area, thousand kernel weight, grain yield, biological yield, % harvest index and straw yield ($P<0.05$, Supplementary Table 3). The interaction between cultivar and mycorrhizal inoculation was also significant ($P<0.05$). All treatments as well as interactions among them influenced the number of grains per plant and grain yield components (Supplementary Table 3).

Grain number for barley cultivars

There was a significant effect of both ZnO application and mycorrhizal inoculation and interactions between them for grain number for both barley cultivars ($P<0.01$; Supplementary Table 3). Mycorrhizal inoculation generally increased the number of grains regardless of Zn applications (Supplementary Table 3). Inoculation with *F. mosseae* and ZnO increased the number of seeds per plant in Julgeh more than in Yousef. The greatest number of seeds (417 m^{-2}) was recorded for Julgeh inoculated with *F. mosseae* with addition of nano+ordinary ZnO, whereas the greatest seed number (378.6) for Yusuf occurred with the combination of *R. irregularis* inoculation and nano+ordinary ZnO application (Table 3).

Flag leaf area for barley cultivars

Foliar Zn application and mycorrhizal inoculation significantly influenced flag leaf area in both barley cultivars ($P<0.01$; Supplementary Table 3). For flag leaf area, only the interaction between cultivar and mycorrhizal inoculation was significant ($P<0.01$; Supplementary Table 3). While mycorrhizal inoculation significantly increased flag leaf area in both cultivars, the response in Julgeh was greater than that in Yousef (Table 3). The greatest area of flag leaf was recorded for Julgeh inoculated with *F. mosseae* and sprayed by nano+ordinary ZnO. The least leaf area was observed in Yousef with neither Zn nor mycorrhizal inoculation (Table 2). The strongest correlations were identified between flag leaf area and biological yield (R^2 =0.86, $P<0.001$), followed by grain yield (R^2 =0.65, $P<0.01$). There was a weak relationship between flag leaf area and harvest index (R2 =0.33, $P<0.01$; Table 2).

Yield and yield parameters of barley cultivars for Zn and mycorrhizal treatments

Application of foliar Zn and mycorrhizal fungi significantly increased most yield and yield parameters in both cultivars ($P<0.01$; Supplementary Table 3). For Yusuf treated with *R. irregularis* and nano+ordinary ZnO, yield parameters such as biological yield (751 g) and straw yield (440 g) were most affected. In addition, in three other traits (thousand kernel weight, grain yield and harvest index), Julgeh inoculated with *F. mosseae* and treated with nano+ordinary ZnO responded to a greater extent than did Yusuf (Table 3). There are strong correlations among grain yield and grain number $plant^{-1}$ (R^2=0.53, $P<0.01$; Fig. 3a) and thousand kernel weight (R^2=0.9, $P<0.001$; Fig. 3b).

Discussion

Interactions between P and Zn can lead to Zn deficiency in shoots and grain (Khan et al., 2014) which may be overcome with foliar application of Zn to wheat (Zhang et al., 2012). Our study focused on the comparative response of foliar application of Zn in nano and ordinary forms of ZnO by two barley cultivars in the presence and absence of two commercial inocula of AM fungi. The main purpose of the study was to identify a potential agronomic strategy to enhance the concentration of Zn in barley grain. We investigated the efficacy of treatments on Zn grain concentration, physiological traits, yield and yield components. The strategy of increasing the content of Zn in barley seed, which was the main aim of this experiment, seeks to improve nutritional value and quality to guarantee a positive consequence for production in the next year. All Zn-treated plants showed a significantly positive response compared to untreated control plants which could be explained in terms of the physiological importance of Zn in plant metabolism. Foliar Zn application significantly increased grain Zn concentrations in barley, indicating the high mobility of Zn within plants. Julgeh had a greater concentration of Zn in its grain than did Yusuf under the same treatments, demonstrating genetic variation

Table 1. Soil pH and nutrients in soil samples taken from 0-40 cm depth from the site prior to establishing the field experiment.

Soil Texture	% organic C	pH	EC (mS/cm)	P (ppm)	K (ppm)	Zn (ppm)	Fe (ppm)	Cu (ppm)
Sandy loam	1.11%	7.8	2.04	70	550	0.11	2.1	0.5

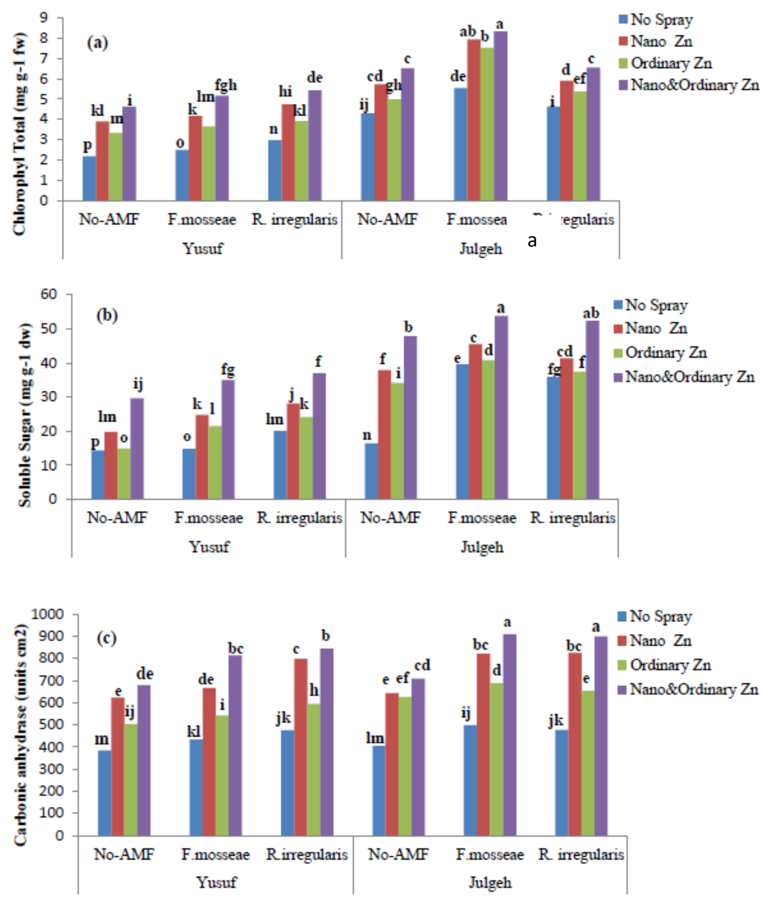

Fig 1. Effects of barley cultivar (Yusuf and Julgeh), inoculation with two commercial inoculants of mycorrhizal fungi (*Funneliformis mosseae* and *Rhizophagus irregularis*) and Zn foliar application on (a) chlorophyll total, (b) soluble sugar and (c) carbonic anhydrase in shoots of barley. Bars represent standard errors.

Table 2. Correlations coefficients between physiological traits and yield parameters for two cultivars of barley, Yusuf and Julgeh with and without inoculation with commercial mycorrhizal inocula and Zn foliar application.

	Ch T (mg g^{-1}fw)	SS (mg g^{-1} dw)	CA (units Cm^{-2})	G PH (mg $units^{-1}$ fw)	GZN (mg kg^{-1}dw)	GN (no $plant^{-1}$)	FLA (cm^2)	TKW (g)	GY (g m^{-2})	BY (g m^{-2})	HI (%)	SY (g m^{-2})
Ch T	1.00	0.00	0.69^{**}	0.62^{**}	0.70^{**}	0.46^{**}	-0.50^{**}	0.42^{**}	0.05^{ns}	-0.67^{**}	0.49^{**}	0.63^{**}
SS		1.00	0.46^{**}	0.43^{**}	0.50^{**}	0.29^{**}	0.26^{*}	0.50^{**}	0.48^{**}	0.38^{**}	0.36^{**}	0.28^{**}
CA			1.00	0.83^{**}	0.90^{**}	0.82^{**}	0.09^{ns}	0.74^{**}	0.64^{**}	-0.08^{ns}	0.84^{**}	0.88^{**}
GPH				1.00	0.87^{**}	0.82^{**}	0.16^{ns}	0.72^{**}	0.59^{**}	0.04^{ns}	0.75^{**}	0.82^{**}
GZN					1.00	0.77^{**}	0.05^{ns}	0.73^{**}	0.62^{**}	-0.08^{ns}	0.78^{**}	0.87^{**}
GN						1.00	0.39^{**}	0.84^{**}	0.53^{**}	0.15^{ns}	0.77^{**}	0.87^{**}
FLA							1.00	0.33^{**}	0.65^{**}	0.86^{**}	0.33^{**}	0.25^{*}
TKW								1.00	0.90^{**}	0.18^{ns}	0.65^{**}	0.72^{**}
GY									1.00	0.00	0.74^{**}	0.66^{**}
BY										1.00	0.17^{**}	-0.0^{ns}
HI											1.00	0.86^{**}
SY												1.00

Physiological traits comprised chlorophyll total (Chl. T), soluble sugar (SS), carbonic anhydrase (CA), grain phytase activity (GPH) , grain zinc (GZn) concentration, and yield parameters such as grain number (GN), flag leaf Area (FLA), plant height (PH), thousand kernel weight (TKW), grain yield (GY), %harvest index (HI), straw yield (SY). * significant at P<0.05; ** significant at P<0.01; ns, not significant.

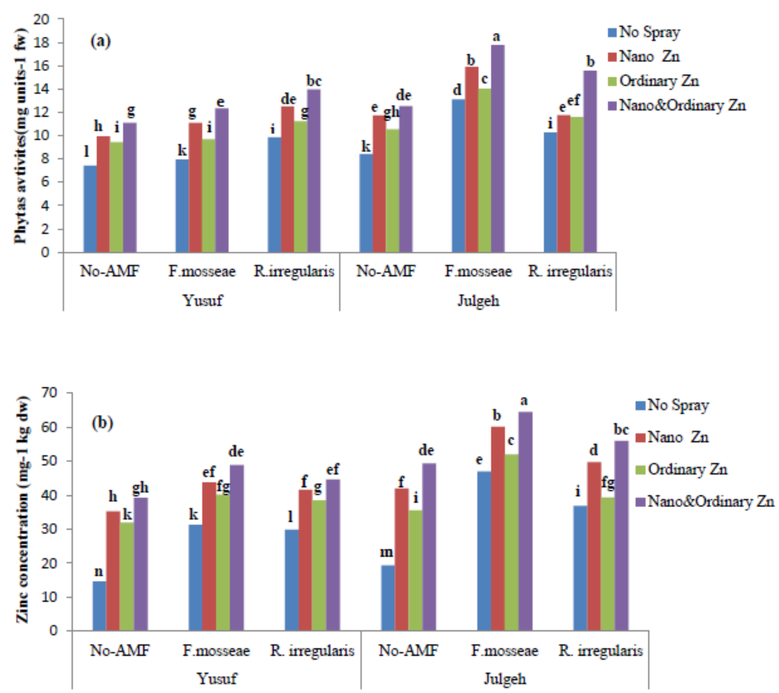

Fig 2. Effects of barley cultivars (Yusuf and Julgeh), inoculation with two commercial inoculants of mycorrhizal fungi (*Funneliformis mosseae* and *Rhizophagus irregularis*) and Zn foliar application on (a) phytase activity and (b) grain Zn concentration. Bars represent standard errors.

Table 3. Effects of barley cultivar, mycorrhizal inoculation and Zn foliar application on shoot and yield parameters.

Barley Cultivar	Mycorrhizal Inoculum	Zn treatments	GN (no plant^{-1})	FLA (cm^2)	TKW (g)	GY (g m^{-2})	BY (g m^{-2})	HI (%)	SY (g m^{-2})
Yusuf	Control	Zn1	232.67^{m}	11.18^{i}	30.53^{o}	226.32^{w}	633.13^{ij}	35.75^{o}	406.82^{de}
		Zn2	275.00^{k}	17.56^{fgh}	33.23^{mno}	267.62t	655.63^{fg}	40.82^{l}	388.02^{g}
		Zn3	248.67^{l}	12.93^{i}	32.13^{no}	245.12^{v}	637.23^{hi}	38.47^{n}	392.12^{fg}
		Zn4	277.33^{k}	19.52^{cde}	34.93^{klm}	281.52^{r}	676.63^{e}	41.60^{kl}	395.12^{efg}
	F. mosseae	Zn1	233.67^{m}	15.65^{ghi}	34.37^{lmn}	260.42^{u}	661.83^{f}	39.35^{mn}	401.42^{ef}
		Zn2	344.33^{h}	28.20^{c}	38.17^{fgh}	301.92^{m}	694.53^{d}	43.47^{i}	392.62^{fg}
		Zn3	301.33^{j}	19.06^{fgh}	34.87^{klm}	284.12^{p}	677.13^{c}	41.96^{jk}	393.02^{fg}
		Zn4	359.33^{g}	33.14^{b}	39.47^{efg}	308.52^{l}	714.53^{c}	43.18^{i}	406.02^{de}
	R. irregularis	Zn1	234.67^{m}	15.12^{def}	34.27^{lmn}	272.52s	706.53^{c}	38.57^{n}	434.02^{ab}
		Zn2	366.67^{a}	27.31^{b}	38.87^{ghi}	317.92^{j}	743.73^{a}	42.75^{ij}	425.82^{bc}
		Zn3	358.67^{g}	19.74^{cde}	35.27^{lmn}	290.22^{o}	729.73^{b}	39.77^{m}	416.02^{cd}
		Zn4	378.67^{a}	33.27^{a}	42.07^{cde}	334.62^{h}	750.63^{a}	44.58^{h}	439.52^{a}
Julgeh	control	Zn1	300.00^{j}	12.89^{i}	35.50^{klm}	267.77^{t}	580.23^{m}	46.16^{g}	312.47^{i}
		Zn2	357.00^{g}	22.33^{def}	36.80^{hig}	297.77^{n}	628.83^{ij}	47.36^{f}	331.07^{h}
		Zn3	327.00^{i}	14.60^{h}	35.90^{ijk}	282.77^{q}	598.03^{kl}	47.29^{f}	315.27^{i}
		Zn4	366.00^{f}	25.80^{cd}	39.20^{fgh}	313.77^{k}	646.73^{gh}	48.52^{e}	332.97^{h}
	F. mosseae	Zn1	302.00^{j}	27.73^{c}	41.50^{cdef}	381.17^{e}	625.03^{j}	56.99^{b}	243.87^{l}
		Zn2	407.67^{b}	35.44^{b}	45.93^{ab}	410.67^{b}	666.83^{ef}	61.59^{a}	256.17^{kl}
		Zn3	390.33^{c}	24.93^{cd}	43.90^{bc}	395.57^{c}	649.13^{g}	60.94^{a}	253.57^{kl}
		Zn4	417.33^{a}	43.59^{a}	48.70^{a}	422.07^{a}	693.93^{d}	61.83^{a}	271.87^{j}
	R. irregularis	Zn1	301.00^{j}	21.12^{ghi}	36.07^{hig}	330.17^{i}	595.93^{l}	55.41^{d}	265.77^{jk}
		Zn2	379.67^{e}	36.87^{c}	40.33^{def}	360.87^{f}	608.73^{k}	59.29^{b}	247.87^{l}
		Zn3	416.67^{a}	23.89^{efg}	38.00^{ghi}	341.87^{g}	602.53^{kl}	56.75^{c}	260.67^{jk}
		Zn4	384.67^{d}	39.11^{b}	43.07^{bcd}	382.17^{d}	627.03^{ij}	60.96^{a}	244.87^{l}
		LSD (0.05)	3.8643	4.7869	3.4328	0.8193	11.662	0.995	12.389

In the same column, values marked with the same letters are similar (P<0.05), whereas those with different letters are significantly different. Zn1= no spraying (control); Zn2 = nano ZnO; Zn3=ordinary ZnO; Zn4 = nano + ordinary ZnO. Number of grains per plant (GN), flag leaf area (FLA), thousand kernel weight (TKW), grain yield (GY), biological yield (BY), % harvest index (HI), straw yield (SY).

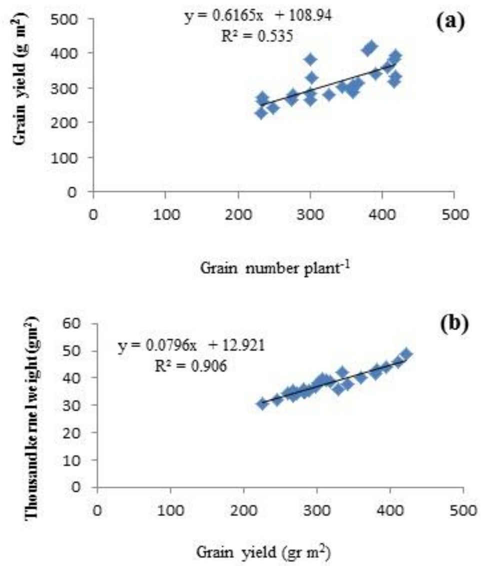

Fig 3. Correlation between grain yield and (a) grain number plant^{-1} and (b) thousand kernel weight of two barley cultivars (Yusuf and Julgeh), inoculation with two commercial inoculants of mycorrhizal fungi (*Funneliformis mosseae* and *Rhizophagus irregularis*) and Zn foliar application.

between cultivars in favor of Julgeh not only in grain Zn content but also in physiological traits and most yield related components. The Zn response in barley grain was comparable to that shown previously for wheat, where foliar Zn application increased grain Zn in wheat cultivars up to threefold under field conditions (Habib, 2009). A similar response in wheat was observed by Cakmak et al. (2010) for foliar application of Zn, even with high P applications.

The greatest Zn concentration occurred with foliar nano Zn followed by foliar application of the combined nano+ordinary forms of Zn which was up to 60 mg kg^{-1}. This is at the upper level of a range from 22 to 61 mg Zn kg^{-1} for barley grain grown under field conditions (Sadeghzadeh, 2013). Furthermore, foliar application of ZnO nanoparticles was most effective in enhancing physiological parameters such as chlorophyll content, total soluble sugar, carbonic anhydrase, grain phytase activity. The effectiveness of foliar spraying with nano ZnO particles may be due to physical properties of the fertilizer, including surface area, leading to enhanced activity, ion adsorption, rapid chemical reaction and complexation (Ramesh, 2014) compared with macro size particles of ordinary ZnO. As in our study, Torabian et al. (2016) illustrated significant positive consequences of applying nano ZnO foliar fertilizer for sunflower leaf area, leaf chlorophyll and Zn content and shoot dry weight compared with the ordinary form of Zn. Our findings are also in agreement with those of Ramesh (2014) where chlorophyll content was greater in nano-ZnO treated rice (*Oryza sativa* L.). Tarafdar et al. (2014) also reported a positive growth response in Pearl Millet following application of nano Zn fertilizer with associated increases in chlorophyll content, total protein soluble and phytase activities.

It appears that aerial spraying of nano Zn offers an improved agronomic biofortification strategy to addressing micronutrient-related malnutrition problems and lack of bioavailability in both barley cultivars. This approach can address crop bioavailability issues by improving phytase enzyme activity. Furthermore, the highest chlorophyll content was observed following foliar application of Zn and mycorrhizal inoculation which is in line with observations of Feng et al. (2002) who reported an increase in chlorophyll content in maize inoculated with *G. mosseae* (now *F. mosseae*) by 32%. It also aligns with the study by Chen et al. (2008) which showed chlorophyll content and chlorophyll a:b ratio in leaves were reduced under Zn deficiency.

In our study, both barley cultivars showed a significant improvement in yield and yield related parameters with application of Zn. While Yusuf invested more in biological yield and straw yield than Julgeh, Julgeh demonstrated more potential to increase grain number, flag leaf area, grain yield, harvest index and thousand kernel weight. The greatest yield parameters in both cultivars performed in order of nano+ordinary Zn, nano ZnO and ordinary ZnO with the control treatment being lowest.

Flag leaf area is highly correlated with yield-related traits, particularly those of thousand-grain weight in cereals (Wang et al., 2011). The area of flag leaf is considered by breeders to be related to higher grain weights and higher yield as achieved by higher photosynthetic rates per unit of flag leaf (Alqudah and Schnurbusch, 2015). Our analysis confirmed these previous studies as larger flag leaf area in Julgeh was correlated with higher grain yield and harvest index. These results concur with findings of Liu et al. (2018) for wheat where larger flag leaf area corresponded to most yield-related parameters, presumably through providing more photosynthetic products for the spikes and grains. Our findings also support observations of Abdel-Ati and Eisa (2015) where significant increases in plant yield and yield parameters followed application of $ZnSO_4$ as a foliar spray on barley in the field. In addition to cultivar differences in response to foliar Zn application, we demonstrated that traits of Julgeh responded more positively to inoculation with *F.*

mosseae, whereas traits of Yusuf responded more positively to inoculation with *R. irregularis*. This was most marked for the nano form of Zn in both cultivars.

There was an increase in activity of phytase in grain of Julgeh following inoculation with *F. mosseae* and foliar application of nano Zn. The maximum activity of this enzyme in Yusuf occurred with the nano form of Zn in combination with *R. irregularis*. While AM fungi can address Zn deficiency (Lehmann et al., 2014; Watts-Williams and Cavagnaro, 2018), there can be an imbalance in absorption of P and Zn from soil following use of P fertilizer (Jiao et al., 2012). Application of Zn to Zn-deficient soils can reduce both absorption and accumulation of P (and thus phytate) in plants (Mousavi, 2011). However, for maize, it was suggested that low availability of Zn in soils with high amounts of P and high pH would be more effectively ameliorated via foliar application of Zn through avoidance of interactions between Zn and P (Tagwira et al., 1993). Thus, benefits of AM fungi in combination with foliar application of Zn could help avoid detrimental interactions between P and Zn in soil (Zhang et al., 2012).

Ercoli et al. (2017) showed that the concentration of Zn in grain of durum wheat inoculated with AM fungi increased under field conditions. However, there can be variation among wheat cultivars in their association with AM fungi (Pellegrino et al. 2015). Indeed, Singh et al. (2012) highlighted adaptation and compatibility of specific species of AM fungi to wheat cultivars. Application of the commercial inoculum of *F. mosseae* used in our study improved Zn absorption and increased barley growth and yield in Julgeh under field conditions. However, across a range of barley traits, Julgeh responded more to inoculation with *F. mosseae*, but Yusuf responded more to inoculation with *R. irregularis*. For the purpose of nutrient security and yield enhancement, Zn foliar especially in the nano form provides a plausible option for ameliorating low Zn concentration in barley grain with potential synergistic benefits from AM fungi.

Materials and methods

Plant materials and treatments

The randomized factorial design used in this field experiment consisted of three factors: two Iranian barley cultivars (Yusuf and Julgeh), two AM fungal treatments (no AM fungi, and commercial inocula of the AM fungi *Funneliformis mosseae* and *Rhizophagus irregularis*) and four ZnO foliar treatments [(control (no Zn), nano-ZnO (2g l^{-1}), ordinary ZnO (2g l^{-1}) and nano ZnO (1g l^{-1}) + ordinary ZnO (1g l^{-1})]. The concentrations of Zn applied were selected based on previous experiment (Esfandiari *et al*. 2016). Both ordinary and nano forms of ZnO which are not soluble in water were suspended directly in deionized water and dispersed by ultrasonic vibration (100 W, 40 KHz) for 40 min and then were prepared at the aforementioned concentrations. Both forms of ZnO used in this experiment were from Iranian Pioneer Nanomaterials Company of Iran. There were three replicate plots per treatment.

Experimental design and set up

The experiment was performed under combined rainfed and irrigated farm conditions at the Agricultural and Governmental research center of Chenaran, Iran (36º 61´ N, 59º 16´ E, altitude 1221m) during the 2015-2016 cropping season. The two barley cultivars were obtained from the Research Center of the Agriculture and Natural Resources of Mashhad, Iran. Seeds were sown at a rate of 350 per m^2 in 10 rows separated by 20 cm in $8m^2$ plots with 3 replications per treatment. There were 72 plots in total. Soil samples (0-40cm) were collected prior to sowing for soil chemical analysis (Table 1).

Basic fertilizers were applied to the site before the start of the experiment based on soil tests. Nitrogen (N) was applied at the rate of 150 kg N ha^{-1} in the form of NH_4NO_3, potassium (K) was applied at the rate of 60 kg K ha^{-1} in the form of K_2SO_4 and P was applied at the rate of 80 kg P ha^{-1} in the form of triple superphosphate.

For Zn treatments, foliar sprays of Zn oxides were applied twice, the first spray occurred at tillering and the second when the grain was at milk stage (early milk development). The average size of nano ZnO was 20 nm and the ordinary ZnO particles with a larger diameter (average 200 nm). Plants were sprayed at sunset to prevent leaf burn, and continued until all leaves were fully impregnated with the solution. According to weather reports, the average rainfall from sowing to harvest in the experimental location was 150 mm with a maximum rainfall in winter. Our two foliar sprays occurred in the autumn and summer seasons and rain did not occur after spraying.

Commercial mycorrhizal inocula were purchased from Turan Biotech in Shahrood, Iran and applied as recommended. A 2 cm layer of inoculum was added to 10 cm deep grooves and covered with 2 cm soil. Seeds were sown and covered with 4 cm of soil. All weeds were manually removed and controlled during growth stages and plants were manually irrigated when required in spring and summer up to maturity. The field plots were well watered and managed in accordance with standard local practices.

Harvesting and plant physiological trait assessment

Two weeks after the second foliar application of Zn when grain was at milky stage, five plants in the same growth stage were randomly selected and marked; the second youngest completely unfolded, fresh and fully developed leaf from the main stem was harvested by hand for comparison of physiological and biochemical traits. Fresh leaf samples (0.3 g) were then analyzed for chlorophyll content (Porra et al., 1989), soluble sugar (Yang et al., 2001), carbonic anhydrase (Gibson and Leece, 1981).

At maturity (210 days after sowing), five random plant samples from each replicate of each treatment were selected for harvest. Samples of aboveground biomass were taken by cutting plants at a height of 1–2 cm. Samples were oven dried at 60 °C for 48 h before assessing dry matter (DM). Grain yield and other yield components, including thousand kernel weight, grain yield, biological yield, harvest index and straw yield were measured.

Grain number spike^{-1}, number of grains plant^{-1}, flag leaf area (m^{-2}), thousand kernel weight (g), grain yield (g m^{-2}), biological yield (g m^{-2}), harvest index (%) and straw yield (g m^{-2}) were evaluated. Phytase activity in grain was assessed (Barrientos et al., 1994), and grain Zn was determined (Westerman, 1990). To estimate Zn concentration, grain samples were ground and digested with a boiling acid mixture (HNO_3 + $HClO_4$) then the concentrations of Zn in the digest were determined on an ARL 3520 inductively coupled plasma (ICP). Descriptions and abbreviations of the 15 measured traits (physiological traits and yield parameters) of barley are presented in Supplementary Table 3.

Measured variables and statistical analysis

Statistical analysis was conducted using SAS software ver. 9.1 and multiple comparisons were made according to Duncan's Multiple Range Test at $P < 0.05$. For data expressed as percentage, a logarithmic transformation was first done. Analysis of variance (ANOVA) and Least Significant Difference (LSD $P < 0.05$) were conducted to compare variables between physiological, chemical traits as well as yield and yield related parameters across growth condition. Pearson correlation coefficients were calculated for the five physiological traits, along with grain yield and yield parameters were implemented by using SAS software.

Conclusion

Foliar Zn application improved barley grain yield and quality (Zn enrichment) when grown in Zn deficient soil under field conditions. There was a positive impact of the nano form of ZnO on physiological and biochemical traits regarding to enhance yield of the two barley cultivars compared. Foliar application of nano ZnO to barley increased grain Zn content in comparison with other Zn sources. Hence, utilization of microelements in the nano form is a potentially effective technology for reducing quantities of fertilizer application. In combination with benefits of AM fungi, foliar Zn application in the nano form could prove to be an effective management practice.

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