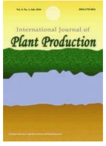

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Coating seeds with endophytic fungi enhances growth, nutrient uptake, yield and grain quality of winter wheat

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Abstract

The aim of this study was to assess whether seed coating with microbial consortium based on the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* BEG72, *Glomus mossae* and *Trichoderma atroviride* MUCL 45632 could improve seedling establishment, yield and grain quality (protein content and mineral composition) of wheat (*Triticum durum* Desf.). As a first step, a laboratory experiment was conducted in a growth chamber to verify the capability of seed coating with endophytic fungi to promote emergence and plant growth of wheat seedlings. Two additional experiments were carried out under open field conditions, to evaluate the effects of coating with beneficial fungi on SPAD index, chlorophyll fluorescence, yield, grain quality and mineral composition of winter wheat. In the growth chamber experiment, 17 days after sowing, the SPAD index, the number of leaves, shoot and root dry biomass of seedlings were significantly higher by 10.0%, 28.6%, 23.1% and 64.2%, in coated as compared to uncoated wheat seeds. In the open field trials, use of the uncoated seeds led to a significant reduction in grain yield by 24.3% and 7.7%, during the first and second growing season, respectively, compared to the coated seeds. Grain quality of wheat, in particular protein content, K, P, Fe and Zn concentrations were improved by AM fungi and *Trichoderma* inoculation. Uncoated wheat plants exhibited a strong variation of yield between the two growing cycles (2.8 and 3.6 t ha^{-1} for 2011-12 and 2012-13, respectively) in comparison to coated seeds (3.7 and 3.9 t ha^{-1} for 2011-12 and 2012-13, respectively). The increase in grain yield and yield stability with coating seed treatment was associated with an increased level of macro and micronutrient uptake, higher SPAD index and photochemical activity of PSII. The application of coated seeds containing *Glomus* and *Trichoderma* can improve the crop performance of wheat in a sustainable way.

Keywords: Arbuscular mycorrhiza fungi; Chlorophyll fluorescence; Mineral composition; SPAD index; *Trichoderma atroviride*; *Triticum durum* Desf.

Introduction

Wheat is one of the major crops contributing approximately to 20% of the world's food requirement, ranking third in production volume (671 mt), after maize (872 mt) and rice (738 mt) and cultivated on 220 million hectares of land worldwide (FAOSTAT, 2013). In Italy, wheat contributed significantly to European Union (EU) economy, occupying 1.9 million hectares area with an annual total production of 7.5 mt. However, its productivity varies from region to region and especially from year to year, because wheat production is highly sensitive to climatic and environmental variables, in particular changing patterns of precipitation, heat and drought stresses (Porter and Semenov, 2005; Karam et al., 2009; Semenov et al., 2014). The variation of yield over time is expected to increase due to climate change, posing a serious danger to food security worldwide (Lobell et al., 2013; Semenov et al., 2014). Moreover, given the depletion of natural resources and development of more regulatory constraints on the use of chemicals in agriculture, farmers tend to produce crops with less synthetic and mineral fertilizers. In the last twenty years, important progress has been made in a number of research disciplines to improve wheat productivity and yield stability under adverse environmental conditions (Reynolds et al., 2009). The introduction of beneficial microorganisms in crop production systems is known as a good sustainable strategy to ensure competitive yields in many crops and improve the resource use efficiency (Singh et al., 2011).

Among the beneficial microorganisms, arbuscular mycorrhizal (AM) fungi are the most widespread root fungal symbionts and are associated with the roots of over 80% of agricultural crops (Ma et al., 2001). AM fungi have been demonstrated to improve soil structure, nutrient uptake by plants in particular phosphorus, maintain and restore soil health and fertility, overcome the detrimental effects of several abiotic stresses such as drought, salinity, toxicity of mineral elements, and adverse soil pH (Al-Karaki et al., 2004; Colla et al., 2008; Cardarelli et al., 2010; Cardarelli et al., 2013; Rouphael et al., 2010). So far the positive effects of AM have generally been attributed to the extension of the host's root apparatus, penetration of substrates and excretion of enzymes by infected roots and/or hyphae and the selectivity of ion uptake (Smith and Read, 1997).

Trichoderma, a mycoparasite that both attacks other fungi and stimulates plant growth, is the most common saprophytic fungus in the rhizosphere (Pill et al., 2009). In addition to their biocontrol activities *Trichoderma* spp. have been found capable of improving solubility of soil micronutrients (Fe, Zn, Cu and Mn) and influencing AM fungi activity (Yedidia et al., 1999; Nzanza et al., 2012). *Trichoderma* spp. can also produce metabolites with hormone activities (Hoyos-Carvajal et al., 2009). The combination of these two beneficial fungi has been reported in several experiments, but with contrasting results (Martinez et al., 2004; Chandanie et al., 2009). The variation in the interaction between AM and *Trichoderma* spp. could be related to the different strains of fungi species (Martinez et al., 2004). Recently, Colla et al. (2015), demonstrated that the combined application of the arbuscular mycorrhizal fungus *Glomus intraradices* BEG72 and *T. atroviride* MUCL 45632 at transplanting act synergistically in improving nutrient uptake, growth and yield of five vegetable crops.

The microbial consortium could also be applied in extensive herbaceous crops for improving their productivity and yield stability over a range of environmental conditions (Khan and Zaidi, 2007; Bahrani et al., 2010; Minaxi et al., 2013). Seed coating is an interesting technology to introduce beneficial microorganisms (Bahrani et al., 2010; Minaxi et al., 2013) and other chemical compounds in the root zone of extensive crops in an efficient and cost effective way (Tavares et al., 2013). Seed coating ensures that the beneficial microorganisms are readily accessible to the root at the critical “early germination” stage, facilitating early, healthy and rapid development and improving nutrient uptake and tolerance to abiotic stresses (Mastouri et al., 2010; Malusa et al., 2012 and references cited therein).

We hypothesized that seed coating of wheat with microbial consortium based on the arbuscular mycorrhizal fungi and *Trichoderma* spp. improve seedling establishment, yield and grain quality. Previous studies reported a good responsiveness of wheat crop to soil/seed inoculation with *Trichoderma* alone (Duffy et al., 1997) or AM fungi in combination with plant growth-promoting rhizobacteria (Bahrani et al., 2010; Minaxi et al., 2013; Rathi et al., 2014). However, to our knowledge no information is available about the effects of seed coating with a combination of compatible strains of *Glomus* spp. and *Trichoderma* spp. on wheat crop performances. In this study, *G. intraradices* BEG72, *G. mossae* and *T. atroviride* MUCL

45632 were selected due to their compatibility as previously reported in vegetables by Colla et al. (2015).

Based on the above considerations, a laboratory experiment was conducted to verify the capability of seed coating with AM fungi (*G. intraradices* BEG72 and *G. mosseae*) and *T. atroviride* MUCL 45632 to promote emergence and plant growth of wheat seedlings. Moreover, two additional experiments were conducted to assess the effects of coating with beneficial fungi on SPAD index, chlorophyll fluorescence, plant growth, yield, grain quality and mineral composition of winter wheat.

Materials and Methods

Experiment 1: short term experiment

The first experiment was carried out in a growth chamber at the University of Tuscia, Central Italy. The growth chamber was programmed to maintain a 12 h photoperiod with corresponding 22 °C light/18 °C night and 65% relative humidity. The average photosynthetic photon flux at the canopy level was 450 μmol m^{-2} s^{-1}. Seeds of wheat (*Triticum durum* Desf., cv. Avispa), were used in all experiments (growth chamber and open field). Seeds were either uncoated or coated with a coating machine. Coating product (Coveron Italpollina, S.p.A., Verona, Italy) contained 300 spores g^{-1} of *Glomus intraradices* BEG72 and 200 spores g^{-1} of *Glomus mosseae* and 3×10^5 CFU g^{-1} of *Trichoderma atroviride* MUCL 45632. The Coveron was selected for use in this study based on plant growth promotion properties of *G. intraradices* and *G. mosseae* and the compatibility of these AM strains with *T. atroviride* MUCL 45632 (Colla et al., 2015). Coating product was applied after dissolution in water at a rate of 1.5 kg t^{-1} of wheat seed.

Treatments were laid out in a randomized complete block design with four replications. Each experimental unit consisted of 84 plants. The experiment consisted of two treatments: uncoated (control) and coated seeds with beneficial fungi. Coated and uncoated seeds were sown in polystyrene trays containing quartz sand. Seedlings were manually fertilized twice a week by a modified Hoagland and Arnon formulation, having the following nutrient composition: 7.0 mmol L^{-1} N-NO_3^-, 1.5 mmol L^{-1} S, 0.2 mmol L^{-1} P, 2.7 mmol L^{-1} K, 5.5 mmol L^{-1} Ca, 1.5 mmol L^{-1} Mg, 20 μmol L^{-1} Fe, 9 μmol L^{-1} Mn, 0.3 μmol L^{-1} Cu, 1.6 μmol L^{-1} Zn, 20 μmol L^{-1} B and 0.3 μmol L^{-1} Mo. The nutrient solution was prepared using deionized water. Electrical conductivity (EC) and the pH of the nutrient solution were 1.8 dS m^{-1} and 6.0, respectively.

The seedlings emergence was calculated based on the total number of emerged seedlings per total number of seeds in each treatment after two weeks from sowing. The mean emergence time (MET) was calculated according to the following formula:

$$MET=\sum n_i\, d_i / n \qquad (1)$$

where, n is the total number of emerged seedlings during the emergence test, n_i is the number of emerged seedlings on day d_i and i is the number of days during the emergence period.

At the end of the first experiment, 17 days after sowing, a chlorophyll meter (SPAD-502, Minolta corporation, Ltd., Osaka, Japan) was used to take readings from the fully expanded leaves. Fifteen leaves per experimental unit were randomly measured and averaged to a single SPAD value for each treatment. On the same date, the number of leaves per seedling was recorded and the seedlings were separated into shoots and roots. The plant tissues were dried in an oven at 80 °C for 72 h for biomass determination.

Experiment 2: open field experiments

The second experiment was performed during the 2011-2012 and 2012-2013 growing seasons from November to July, at the experimental farm of Tuscia University, Central Italy (42° 43’ N, 12° 07’ E; 310 m asl). The soil was a sandy clay loam (32% sand, 33% silt and 35% clay) classified as an Ando-Eutric Cambisol (FAO, 1998), with a bulk density of 1.1 g cm^{-3}, a pH of 7.8, an organic matter content of 1.1% (w/w), exchangeable K at 3,420 mg kg^{-1} and available P at 11 mg kg^{-1}. The monthly air temperature and precipitation compared to the historical means are reported in Table 1.

Table 1. Effect of seed coating treatment on mean days to emergence, total emergence, leaf SPAD index, leaf number, shoot and root dry weight of wheat seedlings at the end of the experiment 1.

Seed coating	Mean days to emergence	Total emergence (%)	SPAD	Leaf number (n. $seedling^{-1}$)	Shoot dry weight (mg $seedling^{-1}$)	Root dry weight (mg $seedling^{-1}$)
No	4.8	87.4	42.1	2.1	9.5	1.4
Yes	3.2	90.1	46.3	2.7	11.7	2.3
Significance[a]	**	ns	*	*	**	*

[a] ns, *, **, nonsignificant or significant at P≤0.05 and 0.01, respectively.

The experimental area was prepared during October 2011 and 2012 with a plow following by disking. The soil was not cultivated during the last two years. In both growing cycles, nitrogen and phosphorus were broadcasted mechanically and incorporated into the upper 15 -cm of soil layer at a rate of 36 kg N ha^{-1} and 40 kg P ha^{-1} as diammonium phosphate (18% of N and 20% of P).

Uncoated and coated wheat seeds containing Coveron were sown on 23 November 2011 and 21 November 2012, using a mechanical plot drill planter with 0.15 m row spacing. The seeding rate was adjusted for a density of 400 seeds m^{-2}, according to the standard management practices adopted in Central Italy. The two field experiments were laid out in a randomized complete block design with five replications in order to compare the following two treatments: a treatment with coated seeds containing Coveron (as reported in experiment 1) and uncoated treatment (control). Each experimental unit consisted of a 28 m^2 plot area, containing 27 wheat rows.

In both growing seasons, granular ammonium nitrate (27% of N) was applied in early March as topdressing at a rate of 350 kg ha^{-1} (94.5 kg N ha^{-1}).

Weed were chemically controlled by one application of pre-plant herbicide ‘Treflan’ (a.i. 480 g L^{-1} of trifluralin) and one application of post-emergence herbicide ‘Manta Duo’ (a.i. 2.5 g L^{-1} of Florasulame and 100 g L^{-1} Fluroxipir). In both treatments, herbicides were applied at the recommended doses by the producers.

At late tillering [84 and 80 days after sowing (DAS) in 2011-12 and 2012-13 growing seasons, respectively] and anthesis (178 and 167 DAS in 2011-12 and 2012-13 growing seasons, respectively), the SPAD index and chlorophyll fluorescence were recorded. SPAD measurements were recorded using the same procedure in experiment 1. The chlorophyll fluorescence was recorded on 15 min dark-adapted leaves on six plants per experimental unit (five leaves per plant) by means of a chlorophyll fluorometer Handy PEA (Hansatech Instruments Ltd, King’s Lynn, UK) with an excitation source intensity higher than 3000 µmol m^{-2}s^{-1} at the sample surface. The minimal fluorescence intensity (F_0) in a dark-adapted state was measured. The maximal fluorescence level in the dark-adapted state (F_m) was induced by 0.8 s saturating light pulse (3000 µmol photons m^{-2}s^{-1}). The maximum quantum yield of open photosystem II (PSII) (F_v/F_m) was calculated as $(F_m-F_0)/F_m$, as described by Maxwell and Johnson (2000).

Six plants per experimental unit were sampled at anthesis growth stage. These samples were taken by a fork, in order to obtain the soil volume under the area occupied by the wheat plants. Roots were gently rinsed to remove soil and subsamples were saved for the determination of AM fungi root colonization and quantification of *Trichoderma*.

Mycorrhizal colonization levels were determined microscopically by the gridline intersect method (Giovannetti and Mosse, 1980) after clearing the roots in 10% KOH solution (wt/vol) and by staining then with 0.05% trypan blue in lactophenol as described by Phillips and Hayman (1970). *Trichoderma* was detected and quantified using serial plating soil dilution on to a *Trichoderma*-selective agar medium (TSM) as described by Elad et al. (1981). Briefly, ten grams of each root/soil sample was suspended in sterilized distilled water to give a dilution of 1:10. Serial dilutions were made to 10-8. Aliquots (10 µL) of each dilution and replicates (four) were spread on to TSM medium in Petri plates. The plates were then incubated at 28 °C for 2-4 days. At the end of the incubation period, fungal colonies of *Trichoderma* were counted and the number of CFU per gram of dry soil was determined (Elad et al., 1981).

Harvest occurred on 6 July 2012 and 14 July 2013. Grain yield was determined in sampling areas of 18 m^2 from the middle rows of the experimental units. The number of grains, 100-seed weight and grain yield were measured. The number of grains was determined by counting grains from all spikes using a seed counter. Grain protein concentration was determined by Kjeltec Analyzer Unit and expressed at dry weight basis.

Dried shoot and grain were ground and 0.5 g of the dried tissues was analyzed for the following macro and micro nutrients: N, P, K, Ca, Mg, Fe and Zn for shoot and K, P, Fe and Zn for grain. Nitrogen was determined by the Kjeldahl method (Bremner, 1965) after mineralization with H_2SO_4. Phosphorus, K, Ca, Mg, Fe, Cu, Zn, Mn and B concentrations were determined in dry ash from tissues burned at 400 °C for 24 h, dissolving the ash in HNO_3 1:20 v/v and assaying the solution obtained by an inductively coupled plasma spectrophotometer (ICP Iris; Thermo Optek, Milano, Italy; Isaac and Johnson, 1998).

Statistical analysis

All data were statistically analyzed by ANOVA using the SPSS software package (SPSS 10 software package for Windows 2001; www.ibm.com/software/analytics/spss). Duncan's multiple range test was performed at p=0.05 on each of the significant variables measured.

Results

Experiment 1: effects of seed coating with beneficial microorganisms on emergence rate and seedling growth of wheat

Coating significantly reduced the mean emergence time of winter wheat by 33.3% as compared to uncoated seeds, whereas no significant difference among treatments was observed for the final percentage of seedling emergence (avg. 88.7%). Strong coating effects on chlorophyll content and seedling growth parameters (Figure 1) were observed in the growth chamber experiment. In fact, the SPAD index, the number of leaves, shoot and root dry biomass were significantly higher by 10.0%, 28.6%, 23.1% and 64.2%, in coated as compared to uncoated wheat seeds (Table 1).

Figure 1. Wheat seedlings uncoated (left) and coated (right) with *Glomus intraradices*, *Glomus mossae* and *Trichoderma atroviride* in the experiment 1.

Experiment 2: performances of wheat plants from coated seeds in field trials

Air temperatures varied during the growth season and between the two consecutive growing seasons. The coldest air temperatures occurred during February dropped to 2.9 and 5.0 °C in 2011-2012 and 2012-2013, respectively (Table 2). In both growing seasons, the air temperature began to rise from March and the mean temperature was always higher than 13 °C from April till July. The total precipitation during the growing seasons of 2011-2012 and 2012-2013 were 313.1 and 899.8 mm, respectively (Table 2), indicating 187% more rainfall in the growth season of 2012-2013 compared to 2011-2012. The rainfall during the second growing season (2012-2013) was higher than the 30-years mean rainfall, whereas the 2011-2012 growing season was much drier than the historical mean.

Table 2. Rain and mean air temperature recorded during the two growing seasons (2011-2012 and 2012-2013) compared to long run data.

Climatic parameters	Years	Month									Total or mean
		Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	
Rain (mm)	2011-12	25.6	45.8	23.7	55.8	0.8	72.8	82.2	3.8	2.6	313.1
	2012-13	204.8	67.7	113.4	107.8	119.8	44.8	126.2	74.9	40.4	899.8
	1984-2013	110.5	77.3	57.2	50.5	57.4	65.7	60.5	42.7	30.1	591.9
Temp. (°C)	2011-12	10.7	8.3	6.2	2.9	11.8	12.6	16.1	22.9	25.2	13.0
	2012-13	12.2	6.1	6.1	5.0	9.0	14.3	15.2	19.7	23.5	12.3
	1984-2013	10.6	7.0	6.2	6.5	9.2	12.0	16.4	20.2	23.4	12.4

The percentage of AM colonization at anthesis growth stage was significantly affected by coating. The percentage of root colonization from coated seeds was higher by 104.3% and 106.3%, for 2011-2012 and 2012-2013 growing seasons, respectively in comparison with the respective uncoated seed treatment (Table 3). Moreover, the mycorrhizal root colonization was clearly higher by 42.4% in the drier season (2011-2012). The number of *Trichoderma* colonies found in the soil and roots in the coating treatment ranged between $5.4\times10^{\wedge4}$ and $1.7\times10^{\wedge5}$, whereas in the control (uncoated) treatment the number of *Trichoderma* colonies was significantly lower and ranged between $1.8\times10^{\wedge2}$ and $2.2\times10^{\wedge3}$ (Table 3).

Table 3. Effect of seed coating treatment on mycorrhizal root colonization and *Trichoderma* spp. population in roots of wheat plants at anthesis during two consecutive growing seasons.

Seed coating	Mycorrhiza root colonization (%)		*Trichoderma* spp. (CFU/g)	
	2011-12	2012-13	2011-12	2012-13
No	23	16	$2.2\times10^{\wedge3}$	$1.8\times10^{\wedge2}$
Yes	47	33	$1.7\times10^{\wedge5}$	$5.4\times10^{\wedge4}$
Significance[a]	***	**	*	**

[a]*, **, ***, significant at $P\leq0.05$, 0.01 and 0.001, respectively.

Coating seeds with *G. intraradices*, *G. mosseae* and *T. atroviride* increased SPAD index during tillering stage by 10.8% and 7.4% in 2011-2012 and 2012-2013, growing season respectively. A similar trend was also recorded during anthesis growth stage since coated seeds displayed increased chlorophyll content by 5.8% and 12.1% in 2011-2012 and 2012-2013 growing season, respectively (Table 4). Moreover, the lowest efficiency of PSII in dark-adapted leaves of winter wheat, measured as F_v/F_m ratio during anthesis, was recorded in the uncoated treatment in comparison to coated seeds (Table 4).

Wheat plants from the coated seeds displayed a significant increase (by 32.1% and 8.3%, during the first and second growing season, respectively) in grain yield, compared to those from the uncoated seeds treatment (Table 5). The lowest seed yield reduction observed in coated in comparison to uncoated treatment was attributed to a reduction in the number of seeds per plant (data not shown) and not to the seed mean weight, (avg. 100-seed weight = 5.2 g). The protein content in wheat grains coming from coated seeds was significantly higher by 6.3% during the 2011-2012, whereas no significant difference among treatments was recorded during the 2012-2013 growing season (Table 5). A similar positive effect of coating seeds was also observed on the mineral composition of winter wheat grains, since in both growing seasons the concentrations of phosphorus, potassium, iron and zinc were significantly higher in coated as compared to uncoated treatment (Table 5).

The concentration of N in leaves of wheat was significantly enhanced by 20% and 8% in 2011-2012 and 2012-2013, respectively when seeds were treated with Coveron (Table 6). The concentration of P significantly increased by 11.4% and 7.7% in the first and second growing season, respectively, with coating in comparison to the control treatment (Table 6). The concentration of K in the leaves of wheat was significantly increased by 12.7% and 10%, respectively when coated wheat seeds were adopted. The concentration of Mg in leaves was only affected during 2011-2012, with the highest values recorded in with coated seeds, whereas no significant difference was observed among coating for Ca concentration (avg. 5.0 g kg^{-1}). Finally, the highest concentrations of micronutrients, in particular Fe and Zn, were observed in the coated treatment (Table 6).

Table 4. Effect of seed coating treatment on SPAD index and maximum quantum use efficiency of PSII in dark-adapted state (F_v/F_m) of winter wheat at tillering and anthesis stages.

Seed coating	SPAD index				Fluorescence			
	Tillering		Anthesis		Tillering		Anthesis	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
No	35.1	36.5	37.8	39.6	0.71	0.81	0.66	0.70
Yes	38.9	39.2	40.0	44.4	0.80	0.85	0.78	0.77
Significance[a]	*	*	*	**	**	ns	**	*

[a] ns, *, **, nonsignificant or significant at P≤0.05 and 0.01, respectively.

Table 5. Effect of seed coating treatment on yield, 100-seed weight, protein content and mineral composition of wheat grains during two consecutive growing seasons.

Seed coating	Grain Yield (t/ha)		100-seed weight (g)		Protein content (%)		K (g kg^{-1})		P (g kg^{-1})		Fe (g kg^{-1})		Zn (g kg^{-1})	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
No	2.8	3.6	5.0	5.3	13.9	14.8	4.2	4.8	3.3	4.3	30.9	37.1	25.8	29.7
Yes	3.7	3.9	5.2	5.5	14.8	15.1	4.7	5.1	4.0	4.7	36.8	39.6	30.5	32.2
Significance[a]	***	*	ns.	ns	*	ns	**	**	***	*	**	*	**	*

[ans], *, **, nonsignificant or significant at $P \leq 0.05$ and 0.01, respectively.

Table 6. Effect of seed coating treatment on mineral composition of durum wheat leaves during two consecutive growing seasons.

Seed coating	N (g kg^{-1})		P (g kg^{-1})		K (g kg^{-1})		Ca (g kg^{-1})		Mg (g kg^{-1})		Fe (g kg^{-1})		Zn (g kg^{-1})	
	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13	2011-12	2012-13
No	23.5	27.8	3.5	3.9	28.4	30.7	4.9	5.0	3.6	4.0	85.3	88.9	24.8	29.7
Yes	28.2	30.0	3.9	4.2	32.0	33.8	5.0	5.2	3.9	4.1	92.0	93.2	30.5	33.2
Significance	**	*	**	*	*	*	ns	ns	*	ns	**	*	**	*

[ans], *, **, nonsignificant or significant at $P \leq 0.05$ and 0.01, respectively.

Discussion

The application of beneficial microorganisms in particular AM fungi is gaining popularity among farmers, because of their potential to increase crop productivity in a sustainable and environmentally friendly way (Singh et al., 2011 and references cited therein). However, studies on the effects of coating winter wheat seeds with potential microbial consortium based on AM fungi and *Trichoderma* are still lacking. The results of the growth chamber experiment demonstrated that coated seeds with Coveron promote earliness of emergence and markedly improved the SPAD index, leaf number, shoot and root dry weight of seedlings (Table 1). This is of outstanding importance for farmers because a successful crop requires high seedling emergence rate and especially strong seedling vigor. Our results are in line with those of Mastouri et al. (2010), who reported that seed treatments with *Trichoderma* are capable of improving seedlings performance. Moreover, Minaxi et al. (2013) found that inoculation of wheat with the AM fungi *G. etunicatum* increased germination of seeds by 13.7%. In a recent study Colla et al. (2015) demonstrated that the application of *G. intraradices* BEG72 and *T. atroviride* MUCL 45632 at transplanting, significantly improved the chlorophyll content (e.g. SPAD index) and the biomass production of five vegetable transplants at early stages of development (e.g. starter effect). Although the results of the first experiment highlighted that the technology for inoculating AM fungi and *Trichoderma atroviride* MUCL 45632 to seed was successful, it was necessary to confirm whether the use of coated seeds with beneficial fungi could also improve crop performance of winter wheat during the whole growing cycle under field conditions.

In both growing seasons (2011-2012 and 2012-2013), the coated seeds co-inoculated with *Glomus* spp. and *Trichoderma atroviride* MUCL 45632 increased grain yield. Co-inoculation of seeds with AM fungi and *Trichoderma* spp. is reported to promote growth in different crops (Calvet et al., 1993; Chandanie et al., 2009; Colla et al., 2015). Colla et al. (2015) observed that combined inoculation of *G. intraradices* BEG72 and *T. atroviride* MUCL 45632 synergistically increased the biomass production of lettuce, tomato and zucchini squash transplants compared with inoculation *G. intraradices* or *T. atroviride* alone. Similarly, Calvet et al. (1993) and Chandanie et al. (2009) demonstrated that co-inoculation of *G. mosseae* and *T. harzianum* had a synergetic influence on plant growth and development of marigold and cucumber plants, respectively. Moreover, coated seeds containing *G. intraradices* BEG72, *G. mossae* and *T. atroviride* MUCL 45632 were capable of maintaining a higher maximum quantum use efficiency of PSII during tillering and anthesis stages (Table 4); the F_v/F_m ratio is widely adopted as an effective tool to quantify the stress damage on the Photosystem II. The chlorophyll content expressed as SPAD index was also higher in coated as compared to uncoated seeds during both phenological stages (Table 4). These results are in agreement with those previously found by Mathur and Vyas (2000) who demonstrated that AM root colonization increased chlorophyll synthesis, which could be associated with a higher net assimilation rate of CO_2 and plant growth. Similarly, inoculation of wheat with *G. fasciculatum* or *G. etunicatum* enhanced shoot and root dry weight, grain yield and mean weight (Bahrani et al., 2010; Minaxi et al., 2013). Moreover, Moubarak and Abdel-Monaim (2011) reported an increase of yield and 1000 kernel weight when wheat was inoculated with *T. harzianum* and *T. viride*.

Furthermore, the grain quality of wheat, in particular protein content, potassium, phosphorus and iron concentration (Table 5), which are particularly important for bread quality, was improved by AM fungi and *Trichoderma* inoculation. The increases in protein content observed in the current work agreed with previous experiments, where the inoculation of wheat with AM fungi and other beneficial microorganisms increased the quality of wheat grains (Khan and Zaidi, 2007; Kumar et al., 2011; Bahrani et al., 2010).

Supposed mechanisms involved in the stimulation of grain yield by coated seeds containing *G. intraradices* BEG72, *G. mossae* and *T. atroviride* MUCL 45632 include beneficial interactions with plant roots. The production of plant growth hormones such as gibberellic and indole acetic acid is one of the mechanisms by which *Trichoderma* spp. can increase grain yield. Colla et al. (2015), reported that *T. atroviride* MUCL 45362 strain used in the current experiment, was capable of producing auxin-like compounds under a wide range of substrate pH (5.5-8.0), leading to an increase in plant rooting (e.g. increased root hair production and deeper roots) which is a desirable trait for seedling establishment contributing in the achievement of high yield (Gorim and Asch, 2012). Moreover Colla et al. (2015), showed that *T. atroviride* MUCL 45362 produced siderophores (hydroxamate and catechol compounds) which are organic compounds able to solubilize micronutrients like iron.

Several researches have also demonstrated that plant growth and yield enhancement by AM fungi is related to the colonization level and to the expansion of external mycelium in terms of length and surface area (Rohyadi et al., 2004). The extension of the host's root apparatus improved the nutrient uptake and translocation (Table 6). Our results are in agreement with those of Cardarelli et al. (2010) and Rouphael et al. (2010) who observed an improvement in nutrient uptake when *G. intraradices* BEG72 was used. The improved nutritional status in the open field experiments (e.g. N, P, K, Fe and Zn) observed in wheat plants coming from coated seeds may be considered as an indirect effect of the beneficial microorganisms. Similarly, Minaxi et al. (2013) reported an increase of N and P in wheat shoots when an inoculum of AM fungi *G. etunicatum* was applied.

The results of the current study also demonstrated, that the enhancement in grain yields due to coated seeds containing *G. intraradices* BEG72, *G. mossae* and *T. atroviride* MUCL 45632 was higher (by 32%) for wheat cultivated in 2011-2012 growing season characterized by low precipitation (313 mm, drier season) as compared to the 2012-2013 growing season (8%, with 900 mm of rain). Our results, are in line with several studies (Al-Karaki et al., 2004; Beltrano and Ronco, 2008; Mastouri et al., 2010 and references cited therein) showing that the greatest advantage of mycorrhizal fungi and *Trichoderma* treatments to plants occurs when they are under stress (e.g. drought and salinity), indicating that these beneficial fungi ameliorate both biotic and abiotic stresses. According to the stress severity hypothesis reported by Bertness and Callaway (1994) and Brooker et al. (2005), the robustness of mutualism is predicted to increase among symbiotically associated organisms when habitat adversity increases for one or both symbiosis partners. Finally, uncoated wheat plants exhibited a strong variation of yield between the two growing cycles (2.8 and 3.6 t ha^{-1} for 2011-12 and 2012-13, respectively) while the yield variation across the two growing cycles was less pronounced when coated seeds were used (3.7 and 3.9 t ha^{-1} for 2011-12 and 2012-13, respectively). The above findings indicated that seed coating with *G. intraradices* BEG72, *G. mossae* and *T. atroviride* MUCL 45632 has the potential to improve yield stability of winter wheat under different environmental conditions.

Conclusions

In conclusion, coating seeds with microbial inoculum containing *G. intraradices* BEG72, *G. mossae* and *T. atroviride* MUCL 45632, enhanced the mean emergence time and shoot biomass of wheat seedlings, through an increase in root dry weight. Our results also demonstrated, that coated seeds with *Glomus* and *Trichoderma* had improved grain quality (protein, P, K and Fe concentration), yield and yield stability among the two growing seasons. The enhancement in grain yields and yield stability in the coated treatment, was associated to a higher chlorophyll concentration, higher photochemical activity of PSII and to a better nutritional status (higher leaf N, P, K, Fe and Zn concentration) of wheat.

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