

Ability of sulfur-oxidising bacteria to hasten degradation of ground rubber particles in soil for release of zinc as a fertiliser to correct deficiency in wheat

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Abstract. Previous research has shown that ground rubber from tyres can be used to supply fertiliser zinc (Zn) for prevention of Zn-deficiency in crops, and that inoculation of the ground rubber with several bacterial species hastens the release of Zn to the soil. We evaluated the ability of several microbial combinations to speed the release of Zn from ground rubber and to decrease soil pH to favour phytoavailability of Zn to crops. In a batch experiment, treatment combinations of two rates of ground crumb rubber (nil or 300 mg kg⁻¹, equal to 0 or 3.4 mg Zn kg⁻¹) and 24 bacterial inoculants were incorporated into a Zn-deficient calcareous soil. In a pot experiment, two wheat cultivars were grown on the soil without or with ground rubber amendment or with equivalent Zn from ZnSO₄ (15 mg kg⁻¹) in combination with two selected microbial treatments. All microbial treatments significantly decreased soil pH at week 3, most notably the inoculant comprising *Rhodococcus erythropolis* and *Acinetobacter calcoaceticus* (RA) + *Pseudomonas putida* P41 (P1) + mixed *Thiobacillus* spp. (Mt). In the presence of tyre rubber, soil pH at week 10 was still significantly lower than the initial value, and soil DTPA-extractable Zn concentration increased until week 6 and then remained unchanged or slightly reduced at week 10. The greatest increase in DTPA-Zn concentration occurred with the RA inoculation. Microbial inoculation treatments were classified by cluster analysis into eight groups based on soil pH and concentrations of iron (Fe) and Zn. Group 8 produced the lowest pH and highest concentrations of DTPA-Fe (average 6.92 mg kg⁻¹) and DTPA-Zn (average 2.67 mg kg⁻¹). Inoculations with RA and with RA + P1 + *T. thioparus* were the most effective in hastening an increase in DTPA-extractable Zn and significantly enhanced Zn uptake by wheat plants, whereas inoculations with *P. putida* P168 and with RA + P2 + Mt were most effective in decreasing soil pH and increasing plant Fe concentration.

Additional keywords: phytoavailable, rubber-biodegrading bacteria, tire rubber, Zn fertiliser.

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Introduction

Accumulation of waste tyre rubber is a considerable environmental challenge in many countries (Adhikari *et al.* 2000; Gieré *et al.* 2004). These solid wastes contain sulfur, zinc oxide (ZnO), stearic acid, carbon black, proprietary additives and bead wires (Steudel and Steudel 2006). An emerging technology for recycling waste rubber is its use as an effective Zn fertilizer source with no risk of cadmium (Cd) contamination in agricultural lands (Chaney 2007; Taheri *et al.* 2011). Ground rubber contains 1–1.5% Zn, organic sulfur and the rubber matrix along with chemicals used to promote vulcanisation and protect the rubber against the environment.

Ground rubber added to Zn-deficient agricultural soils could provide an inexpensive and persistent source of Zn and sulfur as the particles are slowly biodegraded by soil microbes (Newman and Meneley 2006; Khoshgoftarmanesh *et al.* 2012). Taheri *et al.* (2011) observed that the addition of ground rubber increased

DTPA-extractable soil Zn more than commercial fertiliser Zn and the control, but with a slow initial rate of Zn release to the soil because the Zn is within the cross-linked rubber particles (Taheri *et al.* 2011).

One reason for low efficiency of waste tyre rubber as a source of Zn for plants is the very slow release of Zn from the rubber matrix in alkaline soils. In acidic soils, Zn is released more rapidly and contributes to plant uptake of Zn (e.g. Groenevelt and Grunthal 1998). Rubber can be detoxified, devulcanised and degraded by various microorganisms. *Rhodococcus erythropolis* and *Acinetobacter calcoaceticus* DSMZ 590 are recognised as tyre-degrading bacteria. Inoculation with a combination of *R. erythropolis* + *Escherichia coli* + *A. calcoaceticus* was more effective at reducing soil pH and releasing Zn into the soil than inoculation with each of the bacteria alone (Khoshgoftarmanesh *et al.* 2012). *Rhodococcus rhodochrous*, *Corynebacterium* and *Pseudomonas* are able to break down

2-mercaptobenzothiazole (MBT) (Haroune *et al.* 2004), a toxic vulcanisation accelerator additive in rubber (Diepgen *et al.* 2006). *Pseudomonas putida* can degrade MBT and 2-methylthiobenzothiazole (MTBT) (El-Bassi *et al.* 2010) by using benzothiazole, MBT and MTBT as the sole source of carbon, nitrogen and sulfur when grown in minimum media (El-Bassi *et al.* 2010).

Sulfur-oxidising bacteria, specifically *Thiobacillus* spp., can oxidise disulfide linkages in rubber materials, and thereby devulcanise them (Panigrahi and Fung 2009; Jiang *et al.* 2010). Chritiansson *et al.* (1998) introduced *T. ferrooxidans* as the most efficient S-oxidising species in terms of released sulfate. Li *et al.* (2011) found that *T. ferrooxidans* was able to break sulfide bridges between polyisoprene chains and free up reactive sites where new crosslinks could form upon revulcanisation. *Thiobacillus thioparus* was more effective than *T. ferrooxidans* and *T. thiooxidans* at releasing sulfur from ground rubber, probably due to the fact that *T. thioparus* naturally grows at the neutral pH of rubber particles (Holst *et al.* 1998).

Although the ability of *Thiobacillus* spp. and *P. putida* as major rubber-degrading bacteria has been confirmed, no information is available on the effect of these bacteria on release of Zn and iron (Fe) into soil. Bacterial inoculation of ground rubber has been shown to be effective in hastening degradation of rubber and increasing DTPA-extractable Zn and Fe in calcareous soil (Khoshgoftarmanesh *et al.* 2012). Tyre rubber is not considered a source of Fe fertiliser but degradation of rubber in soil may decrease the soil pH and, consequently, result in higher solubility of soil Fe. In the present study, we extended our effort to identify microbial species that could hasten the release of rubber Zn for plant use. We evaluated the ability of *P. putida* and different *Thiobacillus* spp. alone or in combination with rubber-detoxifying bacteria (*R. erythropolis* and *A. calcoaceticus* DSMZ 590) to degrade ground rubber and release phytoavailable Zn into the soil.

Materials and methods

Experimental soil properties

Surface soil (0–30 cm) was collected for the experiment from the research field at the Agricultural Research Station of Rudasht, Isfahan province, Iran (32°29'N, 52°10'E; altitude 1507 m). Considering the critical deficiency level for DTPA-extractable soil Zn (1.0 mg kg⁻¹) (Mortvedt 1985), soil was

severely deficient in available Zn (see Table 1 for selected soil properties). Soil pH was measured in 1:2 soil–water suspension with a digital pH meter (Model 691; Metrohm AG, Herisau, Switzerland). Electrical conductivity (EC) was measured with an EC meter (Model 26 Ohm-644; Metrohm AG) in a soil saturation extract (Rhoades 1982). Organic matter content was determined by the Walkley–Black method (Nelson *et al.* 1996). The CaCO₃ equivalent was determined by neutralising with HCl, and back-titration with NaOH (Black 1965). Available phosphorus content was determined by a colourimetric method, after extraction with 0.5 M NaHCO₃ (Olsen and Sommers 1990). DTPA-extractable Zn, Fe, lead (Pb) and Cd were extracted by using the method of Lindsay and Norvell (1978) and then the elements were measured by flame atomic absorption spectrometry (AAS) with deuterium background correction.

Preparation and analysis of rubber particles

Samples of tyre rubber debris were obtained from the Yazd Tire, Yazd, Iran. After separating the metal wire, scrap tyres were reduced to shreds <10 cm by a slow-speed shredder. The shreds went through three, successively narrower blade shredders to reduce shreds further to <6 cm. The particles were processed to smaller sizes by grinding rolling mills and sieving them on a stainless-steel sieve (<0.3 mm screen). Samples of ground tyre rubber were obtained from Yazd Tire in Isfahan. For analysis, a subsample of sieved ground rubber was ashed in a furnace at 650°C for 6 h and the ash was extracted by using 5 mL 4 M HNO₃. Concentrations of Zn, Fe, Pb and Cd in the solutions were determined by flame AAS (Table 2).

Preparation of bacterial inoculants

Thiobacillus thioparus PTCC 1668 and *Acidithiobacillus ferrooxidans* (basonym *Thiobacillus ferrooxidans*) PTCC 1647 were obtained as live cultures on agar slant, and *Rhodococcus erythropolis* PTCC 1767 and *Acinetobacter calcoaceticus* PTCC 1318 in the form of freeze-dried cultures, from the Persian Type Culture Collection (PTCC). *Pseudomonas putida* P41 and P168 were obtained in the form of freeze-dried cultures from Iranian Research Institute of Soil and Water.

Optimal pH and temperature for *T. thioparus* PTCC 1668 were reported as pH 9 and 30°C (Waksman 1922). Modified thiosulfate medium DSM 486 was used as the liquid medium. This medium contained nutrients (g L⁻¹) KH₂PO₄ (2.0), K₂HPO₄ (2.0), NH₄Cl (0.4), Na₂CO₃ (0.4), MgCl₂ · 6H₂O (0.2) and

Table 1. Selected physicochemical characteristics of the soil examined
OC, Organic carbon; EC, electrical conductivity; TNV, total neutralising value

Texture	OC (g kg ⁻¹)	pH _{water}	EC _e (dS m ⁻¹)	TNV (g kg ⁻¹)	NaHCO ₃ -P (mg kg ⁻¹)	DTPA-extractable elements (mg kg ⁻¹)			
						Zn	Fe	Pb	Cd
Silt loam	0.31	7.88	3.5	22.5	17.5	0.44	4.8	2.9	<0.03

Table 2. Elemental analysis of the ground tyre rubber

Zn (g kg ⁻¹)	S (g kg ⁻¹)	Fe (g kg ⁻¹)	Cu (g kg ⁻¹)	Mn (g kg ⁻¹)	Pb (g kg ⁻¹)	Cd (g kg ⁻¹)	Cr (g kg ⁻¹)
10.1 ± 1.04	29.8 ± 6.41	75.6 ± 10.10	6.11 ± 1.23	25.5 ± 2.14	15.1 ± 5.64	1.99 ± 0.34	9.36 ± 1.02

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (5.0), plus vitamin and trace metals solutions (Abdehagh *et al.* 2011). *Acidithiobacillus ferrooxidans* PTCC 1647 was cultured at initial pH 1.6 in Fe(II)-based medium containing (g L^{-1}) $(\text{NH}_4)_2\text{SO}_4$ (3), KCl (0.1), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), KH_2PO_4 (0.5) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (44.8) (Silverman and Lundgren 1959). FeSO_4 -free medium was autoclaved at 120°C for 20 min and the FeSO_4 solution was separately sterilised by passage through a $0.2\text{-}\mu\text{m}$ filter and added aseptically to the Fe-free medium (Haghshenas *et al.* 2009). *Rhodococcus erythropolis* PTCC 1767, *A. calcoaceticus* PTCC 1318, and *P. putida* P41 and P168 were cultured in a liquid medium containing peptone and meat extract (Khadem Haghighat *et al.* 2003). A 0.4-mL volume of liquid medium (peptone 5 g L^{-1} , meat extract 3 g L^{-1} , deionised water 1 L) was added to each freeze-dried culture, and after homogenising, the suspensions were transferred to vials containing 20 mL liquid solution. The vials were incubated for 3–5 days at 25°C under dark conditions inside a shaker incubator. A 1-mL sample of each liquid medium was transferred to 200 mL liquid media and stored at the above mentioned conditions. Culture purity was checked by transferring a few drops of the suspensions to plates containing solid culture media (15 g L^{-1}) and incubating them at the optimum temperature for growth of each bacterium.

Batch experiment

After preparing fresh bacterial inoculants, bacteria were collected by centrifugation at $5000g$ for 15 min at 10°C . The pelleted bacteria were then suspended in water and adjusted to $\sim 5 \times 10^7$ cells mL^{-1} before mixing with the ground rubber. Inoculants (1 mL) were added to the ground rubber (300 mg kg^{-1} , equal to 3.4 mg Zn kg^{-1}) before mixing with the soil. In addition, a set of soil samples received inoculant treatments without ground rubber (0 mg Zn kg^{-1}). The 24 bacterial inoculation treatments used are listed in Table 3. The soils were incubated at 25°C , with their moisture kept at 70% field capacity using deionised water. Subsamples of soil were collected from each treatment at 1, 3, 6 and 10 weeks after inoculation to investigate changes in soil pH and available concentrations of Fe and Zn over time. Soil pH and DTPA-extractable Zn, Fe, Pb and Cd were measured by flame AAS as described. The serial-dilution agar-plating technique was used at 3 weeks to enumerate the survival of bacterial cells (Cappuccino and Sherman 2001).

The experiment was set up in a quadruplicate, randomised, complete block design with a split-plot arrangement. The main plot was Zn treatment and the subplot was microbial inoculation treatment. Analysis of variance was conducted using the PROC GLM procedure of SAS version 8 (SAS Institute, Cary, NC,

Table 3. Effect of ground tyre rubber (TR) and microbial inoculation on soil pH and DTPA-extractable concentrations of Zn and Fe at week 3
Af, *Acinetobacter ferrooxidans* PTCC 1647; Tt, *Thiobacillus thioparus* PTCC 1668; Mt, mix of *Thiobacillus* species; P1, *Pseudomonas putida* P41; P2, *Pseudomonas putida* P168; RA, *Rhodococcus erythropolis* PTCC 1767 and *Acinetobacter calcoaceticus* PTCC 1318

Microbial inoculation	Soil pH			DTPA-Zn (mg kg^{-1})			DTPA-Fe (mg kg^{-1})		
	–TR	+TR	Mean	–TR	+TR	Mean	–TR	+TR	Mean
No inoculation	7.95	7.92	7.94	0.66	2.19	1.42	4.66	4.79	4.73
Af	7.84	7.76	7.80	0.80	2.44	1.62	5.15	6.23	5.69
Tt	7.83	7.65	7.74	0.82	2.02	1.42	4.82	6.76	5.79
Mt	7.84	7.75	7.80	0.63	2.54	1.59	5.15	6.28	5.72
P1	7.85	7.78	7.81	0.83	2.41	1.62	5.29	6.38	5.84
P1 + Af	7.82	7.77	7.80	0.83	2.26	1.55	5.28	6.17	5.73
P1 + Tt	7.82	7.70	7.76	0.74	2.25	1.50	4.74	6.43	5.59
P1 + Mt	7.83	7.72	7.78	0.91	2.50	1.70	5.34	6.82	6.08
P2	7.79	7.61	7.70	0.78	2.67	1.72	5.67	7.17	6.42
P2 + Af	7.88	7.85	7.86	0.74	2.65	1.69	5.89	6.09	5.99
P2 + Tt	7.91	7.86	7.88	0.58	2.10	1.34	5.78	6.21	6.00
P2 + Mt	7.87	7.85	7.86	0.94	2.16	1.55	5.87	6.12	6.00
RA	7.86	7.80	7.83	0.83	3.19	2.01	5.41	5.87	5.64
RA + Af	7.84	7.81	7.83	0.71	2.47	1.59	5.53	5.79	5.66
RA + Tt	7.84	7.81	7.83	0.77	3.06	1.91	5.62	5.83	5.73
RA + Mt	7.84	7.75	7.80	0.78	2.82	1.80	5.58	6.05	5.81
RA + P1	7.79	7.77	7.78	0.76	2.68	1.72	5.67	6.39	6.03
RA + P1 + Af	7.77	7.71	7.74	0.74	2.53	1.64	5.84	6.74	6.29
RA + P1 + Tt	7.78	7.78	7.78	0.75	2.99	1.87	6.02	6.28	6.15
RA + P1 + Mt	7.75	7.63	7.69	0.75	2.61	1.68	5.81	6.87	6.34
RA + P2	7.80	7.82	7.81	0.91	2.73	1.82	5.32	5.68	5.50
RA + P2 + Af	7.77	7.71	7.74	0.81	2.77	1.79	6.03	6.40	6.22
RA + P2 + Tt	7.79	7.74	7.77	0.73	2.73	1.73	6.39	6.87	6.63
RA + P2 + Mt	7.77	7.58	7.68	0.59	3.00	1.80	5.45	7.04	6.25
Mean	7.83	7.76	7.79	0.77	2.57	1.67	5.51	6.30	5.91
l.s.d. ($P=0.05$)									
Inoculation		0.02			0.09			0.29	
Inoculation \times TR		0.03			0.13			0.41	
TR		0.01			0.03			0.08	

USA). Means separation was performed by Fisher's protected least significant difference (l.s.d.) method at a significant level of $P=0.05$.

Cluster analyses were performed for each soil parameter (soil pH, DTPA-extractable Zn and Fe) and for all three parameters combined at week 3 by using the Euclidean distances between treatments, as described by Johnson and Wichern (2002). The results of the analysis for each parameter were used to present average results over time for groups of treatments, and the results of the hierarchical clustering procedure for the three parameters combined were displayed graphically with a tree diagram.

Pot experiment with wheat

Based on the results of the batch experiment (inoculations with *R. erythropolis* and *A. calcoaceticus* (RA) and with RA + *P. putida* (P1) + *T. thioparus* (Tt) had the greatest effect on soil pH and available concentrations of Fe and Zn), these microbial treatments were chosen for the pot experiment. A bulk soil sample (the same soil used in the batch experiment) was air-dried, thoroughly mixed, sieved to <8 mm, and sterilised by autoclaving at 121°C for 1 h (Trevors 1996). Soil was mixed with either ground rubber (300 mg kg⁻¹, applied as <0.3-mm-diameter particles) or ZnSO₄ · 7H₂O (15 mg kg⁻¹), each equal to 3.4 mg Zn kg⁻¹. The Zn amendments were inoculated with RA or RA + P1 + Tt, or were uninoculated. In addition, a set of soil samples received the inoculant treatments without Zn amendment. Treatments were thoroughly mixed with the soil and incubated moist (near water-holding capacity) at 25°C for about 1 week before seeding.

Homogenised soil samples weighing 800 g were placed in polyethylene pots. Fertiliser was applied based on recommended rates (Milani *et al.* 1998) and mixed thoroughly with soil before planting. Triple superphosphate was applied at 15 kg phosphorus ha⁻¹ and urea at 200 kg nitrogen ha⁻¹. Potassium (K) and Fe were applied as K₂SO₄ and Fe-EDTA at 100 kg K and 20 kg Fe ha⁻¹. Pots were maintained near water-holding capacity with frequent watering to weight.

Seeds of a Zn-efficient bread wheat cultivar (*Triticum aestivum* cv. Roshan) and a Zn-inefficient durum wheat cultivar (*Triticum turgidum* subsp. *durum* cv. Arya) were surface-sterilised with 70% ethanol (v/v) for 30 s followed by 6% sodium hypochlorite for 15 min. They were then rinsed thoroughly with sterile water and germinated in quartz sand moistened with distilled water. These cultivars have widespread use in Iran. Wheat seedlings were then transferred to the pots of prepared soil and grown for 40 days. At harvest, shoots were cut at 2 cm above the soil surface and weighed; roots were removed and washed in tap water before weighing. Dry masses were recorded after drying root and shoot samples in an oven for 48 h at 70°C.

Dried plant samples (~0.50 g) digested in an APCU-40 75-mL TFM Teflon microwave vessel (START D microwave digestion system; Milestone Srl, Sorisole, Italy), using 5 mL HNO₃ and 3 mL H₂O₂, and then filtered through Whatman No. 42 filter paper, transferred to 50-mL volumetric flasks, and diluted with deionised, distilled water. Elements were analysed by using flame AAS with deuterium background correction.

The experiment was set up as a quadruplicate, completely randomised design with a factorial arrangement. All data were subjected to analysis of variance and means were compared using Fisher's protected l.s.d. ($P<0.05$).

Results

Changes in soil pH and DTPA-extractable Zn and Fe concentrations over time as affected by incorporated ground rubber with various microbial inoculants

For each soil parameter, a cluster analysis was used to classify microbial treatments into different groups based on their effects; the average effects of individual microbial treatment groups on soil pH and DTPA-extractable concentrations of Fe and Zn over time are presented in Figs 1–3.

All microbial inoculation groups significantly reduced soil pH at week 3, although the amount varied among the inoculants (Fig. 1). The greatest reduction in soil pH was found for the RA + P1 + Mt treatment (Group 4, Fig. 1). Group 1 had the smallest effect on soil pH, followed by Groups 2 and 3. Application of tyre rubber resulted in a decrease in soil pH that varied depending on microbial treatment and exposure time; with application of ground rubber, soil pH at week 10 remained significantly lower than initial soil pH. In the inoculant-free tyre-rubber treatment, soil pH slightly decreased at week 3 and then remained unchanged until week 10.

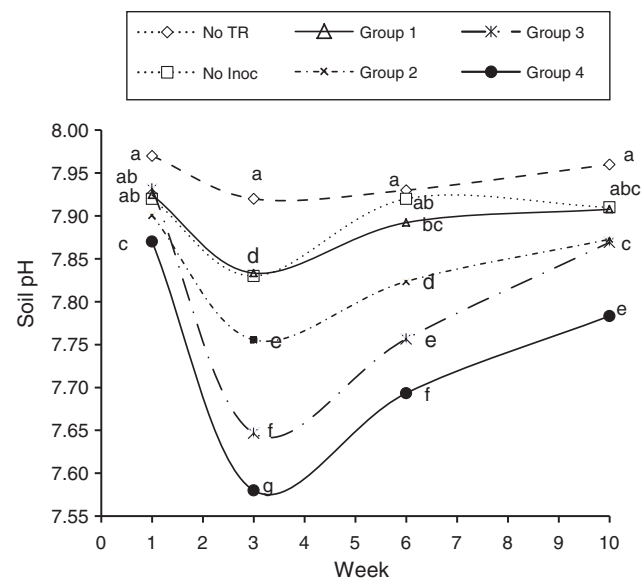


Fig. 1. Changes in soil pH over time as affected by addition of ground tyre rubber and microbial inoculation. At a given time-point, means with the same letter are not significantly different ($P>0.05$). Treatments grouped according to cluster analysis: No TR, no addition of tyre rubber; No Inoc, no microbial inoculation: Group 1—RA; Group 2—P2 + Mt, P1 + Af, P2 + Tt, Af, RA + Af, P1, P1 + Mt, RA + P1 + Af + Mt, P2 + Af, RA + Tt, RA + P1, RA + P2 + Tt, RA + P1 + Mt, RA + P2 + Af, RA + P2, RA + P1 + Tt, RA + P2 + Mt; Group 3—P2, Tt, P1 + Tt; Group 4—RA + P1 + Mt. Bacterial codes: RA, *Rhodococcus erythropolis* PTCC 1767 and *Acinetobacter calcoaceticus* PTCC 1318; P1, *Pseudomonas putida* P41; P2, *Pseudomonas putida* P168; Af, *Acidithiobacillus ferrooxidans* PTCC 1647; Tt, *Thiobacillus thioparus* PTCC 1668; Mt, mix *Thiobacillus* spp.

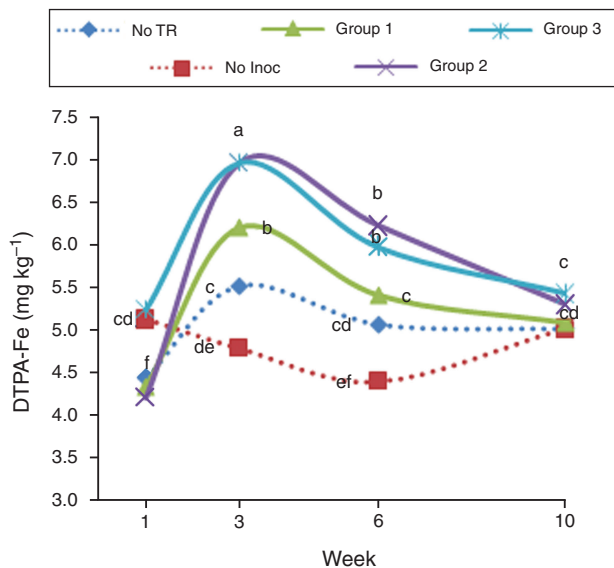


Fig. 2. Changes in soil DTPA-extractable Fe concentrations over time as affected by addition of ground tyre rubber and microbial inoculation. At a given time-point, means with the same letter are not significantly different ($P > 0.05$). Treatments grouped according to cluster analysis: No TR, no addition of tyre rubber; No Inoc, no microbial inoculation; Group 1—RA, P2 + Mt, P1 + Af, Tt, P2 + Tt, P1 + Tt, Af, RA + Af, P1, RA + P1 + Af, P2, Mt, P2 + Af, RA + Tt, RA + P1, RA + P2 + Tt, RA + P2 + Af, RA + P2; Group 2—P1 + Mt, RA + P1 + Mt; Group 3—RA + P1 + Tt, RA + P2 + Mt. Bacterial codes: RA, *Rhodococcus erythropolis* PTCC 1767 and *Acinetobacter calcoaceticus* PTCC 1318; P1, *Pseudomonas putida* P41; P2, *Pseudomonas putida* P168; Af, *Acidithiobacillus ferrooxidans* PTCC 1647; Tt, *Thiobacillus thioarparus* PTCC 1668; Mt, mix *Thiobacillus* spp.

All microbial inoculation treatments resulted in a significant increase in the DTPA-extractable Fe concentration at week 3 compared with no inoculation (Fig. 2). In all inoculated tyre-rubber treatments, DTPA-Fe concentrations increased up to week 3 and thereafter decreased, although they were still higher than their initial concentrations. RA + P2 + Mt and RA + P2 + Tt (from Groups 3 and 1 respectively, Fig. 2) were the most effective inoculation treatments for increasing the availability of Fe in rubber-treated soil.

In the presence of ground rubber, soil DTPA-extractable Zn concentration increased until week 6, then remained unchanged or slightly reduced at week 10 (Fig. 3). The greatest increase in DTPA-Zn concentration was found at the presence of RA microbial inoculation treatment (Group 4, Fig. 3).

Effect of ground tyre rubber and microbial inoculation on soil pH and DTPA-extractable Zn and Fe at week 3

From Figs 1–3, the maximum changes in pH and DTPA-extractable Zn and Fe resulting from the rubber and microbial inoculation treatments were observed at week 3. Therefore, data for that time-point are presented in detail; they show significant variation among the 24 microbial treatments in soil pH and concentrations of DTPA-extractable Zn and Fe (Table 3). For all microbial inoculation treatments, application of ground rubber resulted in greater reduction in pH and increase in DTPA-Zn and -Fe than without ground rubber. At week 3, the highest concentration of Fe and the lowest pH were found in

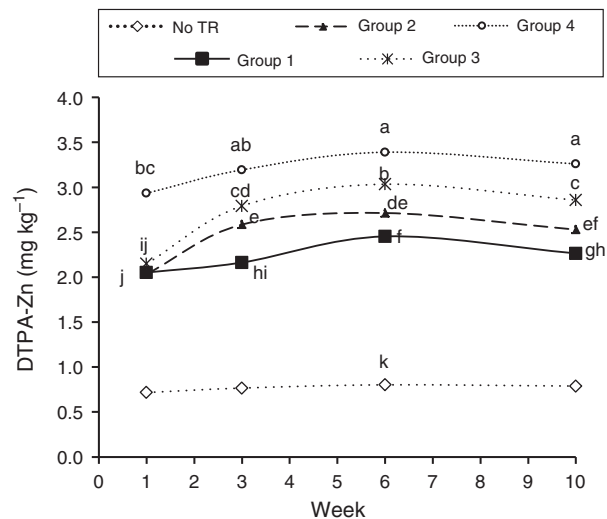


Fig. 3. Changes in soil DTPA-extractable Zn concentrations over time as affected by addition of ground tyre rubber and microbial inoculation. At a given time-point, means with the same letter are not significantly different ($P > 0.05$). Treatments grouped according to cluster analysis: No TR, no addition of tyre rubber; Group 1—no microbial inoculation, P2 + Mt, P1 + Af, Tt, P2 + Tt, P1 + Tt; Group 2—Af, RA + Af, P1, P1 + Mt, RA + P1 + Af, P2, Mt, P2 + Af, RA + Tt; Group 3—RA + P1, RA + P2 + Tt, RA + P1 + Mt, A + P2 + Af, RA + P2, RA + P1 + Tt, RA + P2 + Mt; Group 4—RA. Bacterial codes: RA, *Rhodococcus erythropolis* PTCC 1767 and *Acinetobacter calcoaceticus* PTCC 1318; P1, *Pseudomonas putida* P41; P2, *Pseudomonas putida* P168; Af, *Acidithiobacillus ferrooxidans* PTCC 1647; Tt, *Thiobacillus thioarparus* PTCC 1668; Mt, mix *Thiobacillus* spp.

soil treated with rubber inoculated with P2, RA + P2 + Mt and RA + P1 + Mt. Inoculation of ground rubber with all microbial inoculants except Tt increased DTPA-extractable Zn compared with the non-inoculated treatment, although this increase was smaller for the P2 + Tt and P2 + Mt treatments.

Cluster analysis

Hierarchical cluster analysis classified microbial inoculation treatments into eight groups based on soil pH and concentrations of DTPA-extractable Fe and Zn (Fig. 4). Significant differences were found among the groups (Table 4). Group 2 comprised the non-inoculated rubber treatment and had the highest pH (average 7.92) and the lowest DTPA-extractable Fe (on average 4.97 mg kg⁻¹). Group 5 had the highest DTPA-Zn (average 3.13 mg kg⁻¹) and Group 7 the lowest (average 2.02 mg kg⁻¹), except for Group 1 (no tyre rubber, 0.77 mg kg⁻¹). Microbial treatments classified into Group 8 produced the highest DTPA-Fe (on average 6.92 mg/kg) and high DTPA-Zn (on average 2.67 mg kg⁻¹). Therefore, this group of microbial treatments was selected as the most effective for degradation of rubber and release of Zn and Fe into the soil.

Effect of ground rubber and microbial treatments on wheat yield

Incorporation of ground rubber into the Zn-deficient soil resulted in significant increase in shoot yield of bread wheat (on average 12%) and durum wheat (on average 22%) cultivars

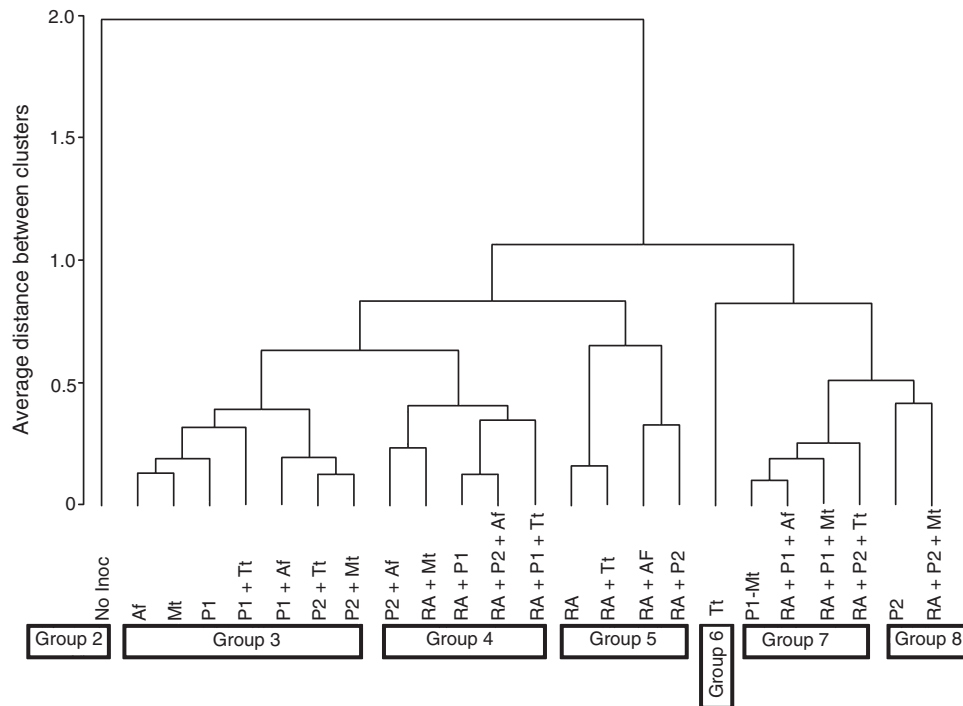


Fig. 4. Cluster analysis of microbial treatments based on soil pH and DTPA-extractable Zn and Fe concentration at week 3. RA, *Rhodococcus erythropolis* PTCC 1767 + *Acinetobacter calcoaceticus* PTCC 1318; P1, *Pseudomonas putida* P41; P2, *Pseudomonas putida* P168; Af, *Acidithiobacillus ferrooxidans* PTCC 1647; Tt, *Thiobacillus thioparus* PTCC 1668; Mt, mix *Thiobacillus* spp.; No Inoc, no microbial inoculation.

Table 4. Mean-squared differences (MS) between clusters of treatments separated based on soil pH and DTPA-extractable Zn and Fe concentration at week 3

TR, Tyre rubber. Within rows, means followed by the same letter are not significantly different at $P=0.05$; * $P<0.05$; ** $P<0.01$

Soil param.	Means							MS within groups	MS between groups
	Group 8	Group 7	Group 6	Group 5	Group 4	Group 3	Group 2 (no inoc.)		
Zn	2.67b	2.02d	2.60b	3.13a	2.78b	2.31c	2.19cd	0.015	5.98**
Fe	6.92a	6.76ab	5.73cd	5.85cd	6.24bc	6.26bc	4.79e	0.11	1.93**
pH	7.66c	7.65c	7.81ab	7.80ab	7.77b	7.78b	7.92a	0.0025	0.024**

compared with the control treatment (Fig. 5). The effectiveness of rubber in increasing wheat shoot dry mass was similar to commercial ZnSO_4 (except with RA microbial treatment for durum wheat, where rubber tended to be more effective). Microbial inoculation of ground rubber in general resulted in significant increases in shoot growth of both wheat cultivars; no significant difference was found between the RA and RA + P1 + Tt treatments.

Effects of ground rubber and microbial inoculation with on plant shoot element concentration

Without inoculation, shoot Zn of durum wheat was not significantly affected by ground tyre rubber or ZnSO_4 fertiliser application treatments (Fig. 6). Without inoculation, shoot Zn of bread wheat tended to be increased by ground tyre rubber and was significantly increased ZnSO_4 . Application of

inoculated ground tyre rubber caused a significant increase in shoot Zn concentration in both wheat cultivars. Wheat plants in the RA-inoculated ground rubber treatment accumulated higher shoot Zn concentration than those grown in the other treatments.

Cadmium and Pb concentrations in shoots of both wheat cultivars grown in all unamended and rubber-amended soils were below the detection limit of AAS ($<0.02 \text{ mg kg}^{-1}$ dry shoot).

Discussion

Application of 300 mg kg^{-1} of ground rubber without microbial inoculation had little effect in reducing soil pH. This agrees with the findings of Brown and Chaney (2000), who found that ground rubber in calcareous soil resulted in no or small changes in soil pH. By contrast, application of microbially inoculated ground rubber significantly reduced soil pH, the decrease

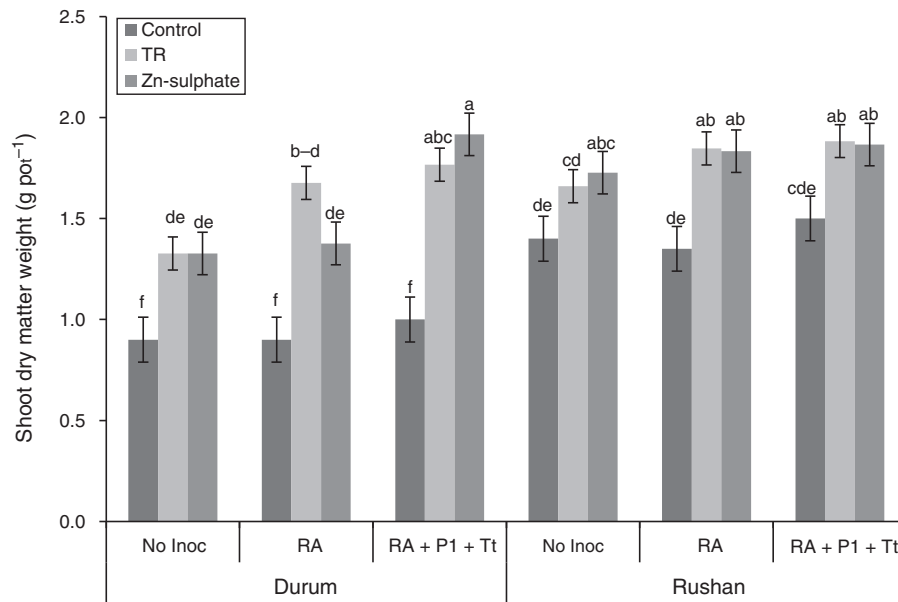


Fig. 5. Effect of ground tyre rubber, ZnSO₄ and two microbial treatments on shoot dry mass of two wheat cultivars. Means with the same letter are not significantly different ($P > 0.05$). TR, Tyre rubber; No Inoc, no microbial inoculation; RA, *Rhodococcus erythropolis* PTCC 1767 and *Acinetobacter calcoaceticus* PTCC 1318; P1, *Pseudomonas putida* P41; Tt, *Thiobacillus thioparus* PTCC 1668.

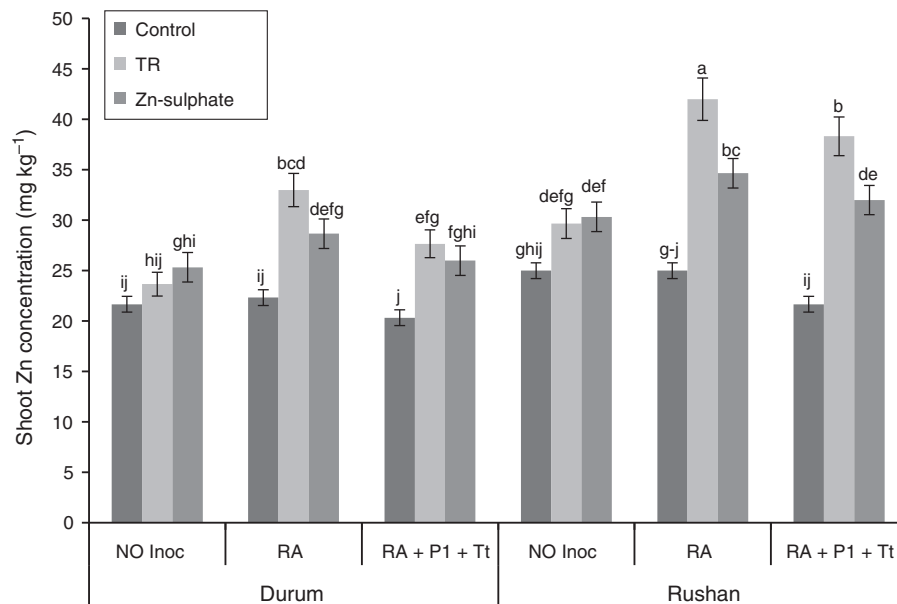


Fig. 6. Effect of ground tyre rubber, ZnSO₄ and microbial treatments on shoot Zn concentration of two wheat cultivars. Means with the same letter are not significantly different ($P > 0.05$). TR, Tyre rubber; No Inoc, no microbial inoculation; RA, *Rhodococcus erythropolis* PTCC 1767 and *Acinetobacter calcoaceticus* PTCC 1318; P1, *Pseudomonas putida* P41; Tt, *Thiobacillus thioparus* PTCC 1668.

depending on microbial treatment and inoculation time. High buffer capacity of the studied highly calcareous soil inhibited large and stable changes in soil pH.

Possible reasons for pH reduction in soil treated with microbially inoculated rubber are the release of sulfuric acid following oxidation of sulfur, and production of CO₂ by

microorganism respiration. Chritiansson *et al.* (1998) reported that *T. ferrooxidans* and *T. thioparus* are able to break S–S bonds at the surface of rubber particles. Li *et al.* (2011) found that *T. ferrooxidans* separated from the soil could break S–S bonds and thereby modify the chemical structure of rubber. Jiang *et al.* (2010) also found that *T. thioparus* oxidised C–S and S–S bonds

and increased the O:S ratio of the rubber. In another study, inoculation with a combination of *R. erythropolis* + *E. coli* + *A. calcoaceticus* was reported to be more effective in reducing soil pH than inoculation with each bacterium alone or non-inoculation treatment (Khoshgoftarmanesh *et al.* 2012).

Application of non-inoculated tyre rubber had little effect in increasing soil DTPA-Fe. By contrast, Taheri *et al.* (2011) and Groenevelt and Grunthal (1998) reported significant increases in soil-available Fe by addition of ground tyre rubber. This variation in response might be due to different properties of the experimental soils and rubber studied. The amount of CaCO_3 in the soil used in the present study was greater than in soils of the previous studies. In addition, the ground rubber applied in our experiment was of larger size and lower Fe content (75.6 mg kg^{-1}) than the rubber applied in the previous experiments (108 mg kg^{-1}). Increasing rubber size decreases the rate of degradation of rubber and its potential effect on soil pH (Taheri *et al.* 2011). Application of microbially inoculated ground rubber significantly increased soil available Fe. In general, an increase in the DTPA-Fe was associated with a reduction in soil pH. Each unit reduction in soil pH increases Fe solubility 1000-fold (Lucena 2000). Therefore, small decreases in pH of rubber-treated soils can effectively increase availability of Fe for plants (Lindsay and Norvell 1978). This should be confirmed in further experiments because we are unsure whether this is relevant to changes in DTPA-Fe after incubation with ground rubber. Any reduction of soil Fe(III) and re-precipitation would give higher DTPA extractability. Air drying a soil increases DTPA extractability of Fe and manganese (Mn) through chemical reaction of dissolved organic matter with Fe- and Mn-oxides during the drying process (Lucena 2000).

In the present study, inoculations with *P. putida* alone (P2) or in combination with *R. erythropolis* and *A. calcoaceticus* (RA + P2 + Mt and RA + P2 + Tt treatments) were more effective than the other microbial treatments in enhancing soil DTPA-Fe. One possible reason for the higher efficiency of *P. putida* with respect to increasing soil Fe solubility is its ability to release Fe-chelating siderophores (Alipour and Sobhanipour 2012). These microbial siderophores can form stable complexes with Fe and can increase solubility of this metal nutrient in soil (Alipour and Sobhanipour 2012).

As expected from previous results, addition of ground rubber, regardless of microbial-inoculation treatment, increased DTPA-Zn concentration. Ground rubber contains ~1% Zn in the form of ZnO. In soils treated with ground rubber, DTPA-Zn concentration was higher than the critical Zn level (0.5 mg kg^{-1}) for wheat (Cakmak *et al.* 1996). The release of Zn from tyre rubber particles (<0.1 mm) into the soil is very slow, such that only 10–40% of rubber Zn was released into the soil after 1 year (Smolders and Degryse 2002). Microbial inoculation hastened the release of Zn from ground rubber into the soil plant-available pool. In the present study, at week 3, microbial inoculation of rubber increased soil DTPA-extractable Zn 4-fold compared with the non-inoculated rubber treatment. Inoculation of tyre rubber with RA (*R. erythropolis* and *A. calcoaceticus*) was more effective than with the other microbial treatments in increasing soil DTPA-Zn. Ability of *A. calcoaceticus* to degrade rubber has been reported by

Bode *et al.* (2001) and Chengalroyen and Dabbs (2013). The *Thiobacillus* species were less effective than the other microbial inoculation treatments in degrading tyre rubber and increasing of DTPA-Zn in soil; however, these species in combination with rubber-degrading bacteria (*R. erythropolis* and *A. calcoaceticus*) significantly enhanced soil DTPA-Zn. It appears that *Thiobacillus* species are sensitive to toxic compounds released from tyre-rubber particles. Toxic chemicals in the tyre rubber, such as zinc oxides and some vulcanising chemicals, slow sulfur-oxidising and rubber-degrading bacteria from naturally breaking it down. Specific environmental conditions are necessary for effective microbial devulcanisation of tyre rubber. Therefore, tyre-rubber-detoxifying bacteria (i.e. *R. erythropolis* and *A. calcoaceticus*) can produce better conditions for *Thiobacillus*. Nowaczyk and Domka (1999) reported that toxic compounds released during degradation of tyre rubber by *T. ferrooxidans* have detrimental effects on growth of this bacterium. *Rhodococcus erythropolis* and *A. calcoaceticus* are able to break down or bio-transform MBT and thereby produce better conditions for activity of other microorganisms. MBT is a well-known toxic additive (Diepgen *et al.* 2006) used as a vulcanisation accelerator in rubber. Metabolites produced during MBT biodegradation are much less toxic (Haroune *et al.* 2004). El-Bassi *et al.* (2010) reported that *P. putida* strain HKT554 is able to break down toxic compounds in tyre rubber such as MBT and MTBT. Our results also showed that the presence of detoxifying bacteria enhanced the efficacy of sulfur-oxidising bacteria in the desulfurisation of tyre rubber.

Cluster analysis of microbial treatments based on their effects on soil pH and DTPA-extractable concentrations of Fe and Zn at week 3 showed that RA and RA + Tt were more effective than the other treatments in enhancing Zn release from the tyre rubber, whereas P2 and RA + P2 + Mt were most effective in reducing soil pH and enhancing Fe concentration in soil treated with tyre rubber. In the pot experiment, addition of 3 mg Zn kg^{-1} in the form of ground tyre rubber inoculated with RA or RA + P1 + Tt increased shoot dry mass and Zn concentration in wheat relative to the control and in some instances, relative to commercial ZnSO_4 . Inoculation of tyre rubber has been reported to be effective in increasing availability of Zn in soil and its uptake by maize (*Zea mays*) (Khoshgoftarmanesh *et al.* 2012). In the present study, the RA treatment was more effective than RA + P1 + Tt in enhancing shoot Zn concentration in wheat grown in rubber-treated soil, although no difference in shoot dry mass of wheat was found between these treatments.

Cadmium and Pb concentrations in shoots of both wheat cultivars were below the detection limit of AAS. This result that is in agreement with our previous findings (Khoshgoftarmanesh *et al.* 2012; Taheri *et al.* 2011), showing that Pb and Cd should not be an issue for using ground rubber as a Zn fertiliser, in strong contrast to several industrial by-products sold as Zn fertilisers that contained high levels of Cd (Mortvedt 1985).

Conclusions

Experiments showed that inoculation of ground rubber with detoxifying bacteria enhanced the efficacy of sulfur-oxidising

bacteria in desulfurisation of tyre rubber and their effect on soil pH and plant-available Zn and Fe concentrations. Cluster analysis of microbial treatments based on their effects on soil pH and DTPA-Fe and -Zn at week 3 showed that RA (*R. erythropolis* PTCC 1767 and *A. calcoaceticus* PTCC 1318) and RA+P1 (*P. putida* P41) +Tt (*T. thioparus* PTCC 1668) were more effective than the other microbial treatments in enhancing Zn release from the tyre rubber, whereas P2 (*P. putida* P168) and RA+P2+Mt (mix of *Thiobacillus* spp.) were the most effective treatments in reducing soil pH and enhancing Fe concentration in soil treated with ground rubber. In the pot experiment, addition of ground tyre rubber (300 mg kg⁻¹) inoculated with RA and RA+P1+Tt increased shoot dry mass and Zn concentration in wheat compared with the control (no added Zn) and, in some cases, commercial ZnSO₄ treatment. The proper environmental conditions for detoxifying bacteria as well as for sulfur-oxidising bacteria could be better defined by further research works.

Conflicts of interest

No potential conflicts of interest is reported by the authors.

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