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1 **Inoculating chlamydospores of *Trichoderma asperellum* SM-12F1 changes arsenic availability**  
2 **and enzyme activity in soils and improves Water Spinach growth**

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18 **Abstract:** Arsenic (As)-contaminated agricultural soils threaten crop yields and pose a human  
19 health risk. Augmentation of exogenous microorganisms exhibiting plant-growth promoting and  
20 As speciation changing shows potential to improve crop growth and change soil As availability.  
21 *Trichoderma asperellum* SM-12F1 exhibiting both traits was developed into chlamydo spores to  
22 improve its persistence in contaminated soils. After inoculation, As availability and enzyme  
23 activity in two types of soils and the growth as well as As uptake of water spinach (*Ipomoea*  
24 *aquatic* Forsk.) were investigated. The results indicated that inoculation significantly improved  
25 water spinach growth in both soils. Inoculating chlamydo spores at 5% significantly increased As  
26 concentration (139%), bioconcentration factor (150%), and translocation factor (150%) in water  
27 spinach grown in Chenzhou (CZ) soils, while no significant change for these in Shimen (SM) soils.  
28 Inoculating chlamydo spores at 5% caused a significant increase (16%) of available As content in  
29 CZ soils, while a significant decrease (13%) in SM soils. Inoculation significantly caused As  
30 methylation in both soils, while significant As reduction merely observed in CZ soils. The  
31 differential changes in available As contents in both soils were attributed to the soil pH, As  
32 fractionations and speciation characteristics. Furthermore, Inoculating chlamydo spores at 5%  
33 significantly improved the activities of  $\beta$ -glucosidase (155%), chitinase (211%), and phosphatase  
34 (108%) in SM soils, while significant decreases in  $\beta$ -glucosidase (81%), phosphatase (54%),  
35 aminopeptidase (60%), and catalase (67%) in CZ soils. Bioaugmentation and As availability change  
36 were responsible for this result. These observations will be helpful for the application of fungal  
37 chlamydo spores in the future bioremediation.

38 **Keywords:** Fungi; Arsenic; Water spinach; Soil enzyme; Speciation; XANES

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40 **1. Introduction**

41 Arsenic (As) accumulates in soil via various natural routes, such as rock weathering and volcanic  
42 activity, as well as from anthropogenic activities that include mining/smelting, crop irrigation and  
43 pesticide/preservative use (Abernathy et al., 2003). There is mounting concern regarding human  
44 exposure to As through soil-plant transfer, in addition to more established exposure risks from  
45 drinking/cooking water supplies. Arsenic's negative effects on human health from chronic  
46 exposure are widespread, but it's most notable as a potent carcinogen (Fayiga and Ma, 2006). In  
47 agronomic settings, As has historically been used extensively as a herbicide and defoliant  
48 (Williams et al., 2005). Impacting on various metabolic processes, As causes physiological and  
49 morphological disorders leading to reduced plant growth (Tripathi et al., 2013; Zhao et al., 2010).  
50 It disrupts also, the regulation of essential nutrients within plant tissues, reducing the overall  
51 quality of crops as a food source (Williams et al., 2007). *In solum*, As can depress soil enzyme  
52 activity, a commonly used metric for biological diversity, ecosystem functioning and overall soil  
53 fertility (Marx et al., 2001; DeForest, 2009), which can also depress plant growth.

54

55 The discovery and effective utilization of microorganisms capable of improved As  
56 tolerance/detoxification combined with plant-growth promotion (PGP) characteristics to mitigate  
57 crop plant exposure to As and enhance productivity are highly prized technologies (Das et al.,  
58 2014). Recent studies showed that arbuscular mycorrhizal fungi inoculation can improve As  
59 tolerance in tomato (Hua et al., 2009), plantain (Orowska et al., 2012), Chinese brake fern (Leung  
60 et al., 2010), and medic (Zhang et al., 2015). Arsenic-resistant bacteria have also been  
61 successfully screened/selected for PGP (Cavalca et al., 2010; Shagol et al., 2014; Ghosh et al.,

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62 2015). It is well known that microbial excretion of hormones and/or enhanced nutrient  
63 supplementation play a role in PGP (Khan et al., 2009; Lampis et al., 2015). Comparatively,  
64 however, more research attention has been paid to the application of PGP by arbuscular  
65 mycorrhizal or bacteria. Far less is known about the potential of filamentous fungi as agents of  
66 PGP for the remediation of As-contaminated soils (Babu et al., 2014a). Filamentous fungi have a  
67 distinct advantage over bacteria because of their high tolerance to As and other  
68 metals/metalloids and their abilities to grow under extreme conditions of pH, temperature and  
69 nutrient availability (Anand et al., 2006). More importantly, some filamentous fungus can be  
70 developed into chlamyospores, with asexual reproduction and thick cell walls that confer  
71 additional protection enabling them to further tolerate contaminated soils (Lewis and Papavizas,  
72 1983). Additionally, fungal chlamyospores can be easily developed into solid powders that are  
73 convenient for storage, and improve the delivery of the inoculum into the soil.

74

75 Some PGP microorganisms can trigger soil As speciation change (ASC), subsequently changing As  
76 phytoavailability. Das et al. (2016) observed that PGP *Bacillus flexus* ASO-6 can oxidize arsenite  
77 (As(III)) and reduce As-uptake by rice and promote plant growth in As-stressed soil. Xu et al.  
78 (2016) found that root or leaflet endophytes exhibiting plant-growth promoting traits can resist  
79 As via arsenate (As(V)) reduction, promote the growth of As-hyperaccumulator *Pteris vittata* and  
80 increase phytoremediation efficiency. These microorganisms that exhibit both PGP and ASC traits  
81 show great potential as biotechnology tools for promoting crop yields, whilst simultaneously  
82 changing plant As uptake. Unfortunately, the study of these PGP-ASC organisms in  
83 As-contaminated soils is scarce.

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85 Soil enzyme activities are known to be affected by As, heavy metals and microbial biomass  
86 (Bhattacharyya et al., 2008). Low enzymatic activities have been observed in soils heavily  
87 contaminated with toxic trace metals (Hagmann et al., 2015). Koo et al. (2012) observed that  
88 water soluble As especially, exerts a strong inhibitory effect on the soil enzyme activities. The  
89 microbial biomass is the major source of enzymes in soil and is highly susceptible to disruption by  
90 As and heavy metal contamination (Boshoff et al., 2014). The augmentation of exogenous  
91 microorganisms capable of As speciation change could enhance the microbial biomass (Tripathi  
92 et al., 2015) as well as labile As content in soil (Wang et al., 2015), which subsequently influences  
93 soil enzyme activities. Thus, enzyme activity analysis can be helpful in assessing the biochemical  
94 quality of As-contaminated soils after microbial inoculation.

95

96 Recently, *Trichoderma asperellum* SM-12F1 has been reported for its abilities of As resistance and  
97 speciation transformation (Zeng et al., 2010; Su et al., 2011, 2012). It has been well reported that  
98 *Trichoderma* greatly contributes to PGP, biological control, and modification of plant metabolism  
99 (Harman et al., 2004). Babu et al. (2014b) observed that *Trichoderma* spp. can enhance host  
100 plant growth through production of Indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylic  
101 acid (ACC) deaminase, siderophores, or acid phosphatase under biotic and abiotic stresses.  
102 Furthermore, *T. asperellum* SM-12F1 was successfully developed into chlamydospores to improve  
103 its persistence in contaminated soils (Wang et al., 2015). In this study, pot experiments were set  
104 up by inoculating with chlamydospores of *T. asperellum* SM-12F1 into two types of As-enriched  
105 soils with different contamination sources (mining vs. industrial). Water spinach (*Ipomoea*

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106 *aquatic* Forsk.), which is a popular vegetable crop in As-contaminated areas of southern China,  
107 was selected as the test plant species. The objectives were to determine: (I) the growth and As  
108 uptake of water spinach after inoculation; (II) the reproduction of *T. asperellum* SM-12F1 and  
109 changes in soil enzymes activities; (III) variances in As availability, fractionation as well as  
110 speciation in soils after inoculation. To our best knowledge, this is the first report to exploit the  
111 application of fungal chlamydo spores capable of plant-growth promotion and As speciation  
112 transformation in As-contaminated soils.

113

## 114 **2. Materials and methods**

### 115 *2.1 Preparation of the chlamydo spores of T. asperellum SM-12F1 and soil samples*

116 The As-resistant fungal strain, *T. asperellum* SM-12F1, was isolated from a slag heap near the  
117 realgar mine in Shimen county of Hunan province, China. The speciation transformation of As by *T.*  
118 *asperellum* SM-12F1 had been well investigated (Zeng et al., 2010; Su et al., 2011, 2012).  
119 Following our previous procedure (Wang et al., 2015), *T. asperellum* SM-12F1 was successfully  
120 cultivated into chlamydo spores via a chlamydo spores production medium and then developed  
121 into solid powder after grinding the air-dried culture residue. The pH of the chlamydo spores  
122 powder (5.78) was determined potentiometrically at a 1: 2.5 ratio of powder to ultrapure water  
123 prepared by using Milli-Q water purification system (Millipore Corporation, USA). No As in  
124 chlamydo spores powder was detected out using hydride generation atomic fluorescence  
125 spectrometer (HG-AFS 9120, Titan instrument, Beijing, China).

126

127 Two types of experimental soils were collected from the As-contaminated field soils in Chenzhou

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128 City (CZ soils) and Shimen County (SM soils) of Hunan province, China, respectively. The former  
129 (Calcari-Leptic Cambisol) developed from the limestone is adjacent to a timber yard where  
130 arsenide is often used in wood preservation, while the latter (Alumi-Plinthic Acrisol) developed  
131 from quaternary red clay is located at the downstream of a realgar mine. Both sampling sites  
132 were contaminated after As enriching soils via flood moving. After air-dried, grounded, and  
133 passed through the 2-mm sieve, the experimental soils were mixed thoroughly. The  
134 physic-chemical properties of soils are listed in Table S1 (in the Supplementary Data).

135

#### 136 *2.2 Pot experiment*

137 The pot experiment was conducted in a glasshouse of Chinese Academy of Agricultural Sciences.  
138 For each experimental soil (SM and CZ), chlamyospores powder ( $1.2 \times 10^7$  cfu  $g^{-1}$  obtained via the  
139 dilution plate method) of *T. asperellum* SM-12F1 with three levels (m/m) of 0% (CK), 1.0%, or  
140 5.0% were inoculated. In order to provide enough carbon and nitrogen sources for fungal survival,  
141 glucose (2.0%) and asparagines (0.5%) were spiked into each soil (Sneh et al., 1984). Furthermore,  
142 each soil received the same amount of chemical fertilizers ( $0.72 \text{ g kg}^{-1}$  of  $\text{CO}(\text{NH}_2)_2$ ,  $0.32 \text{ g kg}^{-1}$  of  
143  $\text{KH}_2\text{PO}_4$ , and  $0.54 \text{ g kg}^{-1}$  of  $\text{K}_2\text{SO}_4$ ) to meet the demands of plant growth modified from the report  
144 of Hseu et al. (2013). After complete mixing, each soil was transferred into a pot. Four  
145 replications were run for each inoculation level. Six seeds of water spinach purchased from  
146 Beijing Ju Hong Seed Technology co., LTD were surface sterilized with  $\text{H}_2\text{O}_2$  (5%) and then sown in  
147 each pot. Subsequently, the seedlings were thinned to three per pot after germination. Soil  
148 moisture content was maintained at 60% of field capacity during the germination and then  
149 adjusted to field capacity after thinning. The temperature in the glasshouse was maintained at



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150 30 °C during the daytime and 25 °C at night. All pots were arranged in a completely randomized  
151 design.

152

### 153 *2.3 Biomass analysis and survival number of T. asperellum SM-12F1 determination*

154 Water spinach was harvested 2 months after sowing. The height of water spinach was measured  
155 by using a stainless steel ruler with accuracy of 0.01cm. The shoot and root of water spinach were  
156 separated and subsequently weighted after being dried at 65 °C for 48 h. The plant samples were  
157 ground using a stainless steel grinder. For each treatment, fresh soils samples were harvested and  
158 divided into three sub-samples. The first (20.0 g) of which was used to determine the survival  
159 number of *T. asperellum* SM-12F1 using the dilution plate method and the selective medium  
160 (Supplementary information Fig. S1) modified from the descriptions of Elad and Chel (1983) and  
161 Papavizas (1982). The second (10.0 g) was immediately stored at -20 °C for As speciation analysis.  
162 Finally, the last portion was air-dried and then ground to pass 2 mm sieve for subsequent  
163 analysis.

164

### 165 *2.4 Analysis of As contents in soil or plant samples*

166 For plant samples, each shoot or root sample of 1.000 g was digested by mixed HNO<sub>3</sub> of 20 ml,  
167 H<sub>2</sub>SO<sub>4</sub> of 1.25 ml, and HClO<sub>4</sub> of 1 ml until the digestion solution was clear (PRC National Standard,  
168 GB/T 5009.11-1996). After filtration and volume fixation at 50 ml, the total As content was  
169 measured using HG-AFS. During HG-AFS analysis, the mixture of 1% ascorbic acid, 1%  
170 thiocarbamide, and 3% hydrochloric acid was used to preliminarily reduce sample As into As(III).  
171 The mixture of 0.5% potassium hydroxide and 1% potassium borohydride was used to further

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172 reduce As(III) into AsH<sub>3</sub> and subsequently As content was determined. 3% hydrochloric acid was  
173 used as sample carrier. In order to characterize As uptake and assimilation patterns in water  
174 spinach, bioconcentration factor (BCF) and translocation factor (TF) were measured. The BCF of  
175 As in water spinach was calculated by the ratio of As concentrations in shoot and soil (Zhuang et  
176 al., 2007). The TF of As was calculated from the ratio of As concentrations in shoot and root (Yoon  
177 et al., 2006).

178

179 For soil samples, soil available As was extracted with 0.5 M NaHCO<sub>3</sub> (Woolson et al., 1971). A soil  
180 sample of 5.00 g was suspended in 0.5 M NaHCO<sub>3</sub> of 50 ml and then shaken for 2 h at room  
181 temperature. Subsequently, the soil suspension was filtered before As determination by HG-AFS.  
182 An improved sequential extraction proposed by Wenzel et al., (2001) was adopted to determine  
183 As fractionation. The five sequential extraction steps were assumed to correspond respectively  
184 to non-specifically sorbed As (F1), specifically sorbed As (F2), As associated with amorphous and  
185 poorly-crystalline hydrous oxides of Fe and Al (F3), As associated with well crystallized hydroxides  
186 of Fe and Al (F4), and residual As (F5). Additionally, soil pH was also measured potentiometrically  
187 at a 1:2.5 ratio of soil to H<sub>2</sub>O after 1 minute of shaking.

188

189 *2.5 In situ analysis of As speciation in soils using in-situ X-ray absorption near edge structure*  
190 *(XANES)*

191 Arsenic speciation analysis of fresh soil samples using in-situ XANES was conducted at beam line  
192 15U1 of Shanghai synchrotron radiation facility. Sub-samples of fresh soil, after defrosting at  
193 room temperature, were fixed to Mylar membranes (thickness of 6 μm) that was fixed onto a

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194 sample table for analysis. For each sample, three sites were randomly selected for XANES analysis.  
195 Each site was scanned for 6 s using a spot size of  $3.18 \times 2.56 \mu\text{m}^2$  from 11,850 to 11,900 eV with a  
196 0.5 eV step size. To correct for the effect of the synchrotron radiation beam flux variation on  
197 signal intensity, the fluorescence intensity was normalized to the incident X-ray intensity, which  
198 was monitored using an ionization chamber located in front of the K-B mirror modulating the size  
199 of the beam (Zheng et al., 2011). The corrected fluorescence intensity was used to estimate the  
200 relative elemental content. During XANES analysis, As standard compounds were prepared with  
201 high-purity (>94.5%) chemicals including As(III),  $\text{NaAsO}_2$  (Riedel-de Haen AG, Seelze-Hannover,  
202 Germany); As(V),  $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$  (Dr. Ehrenstorfer, Germany); MMA,  $\text{CH}_4\text{AsNaO}_3$  (Dr.  
203 Ehrenstorfer, Germany); and DMA,  $\text{C}_2\text{H}_6\text{AsNaO}_2$  (Dr. Ehrenstorfer, Germany).

204

#### 205 *2.6 Analysis of soil enzymes using microplate fluorimetry*

206 Activities of hydrolase enzymes such as  $\beta$ -glucosidase, aminopeptidase, and acid phosphatases  
207 are the keys controlling the availabilities of C, N, and P in soil, respectively (Rejmánková and  
208 Sirová, 2007). Chitinase is essential in the mineralization of N from chitin as a main component of  
209 fungal cell wall (Olander and Vitousek, 2000). Catalase is one of the important antioxidant  
210 enzymes contributing to the detoxification of reactive oxygen species generated due to heavy  
211 metal stress (Patel et al., 2016). In this study, the activities of the  $\beta$ -glucosidase, chitinase, and  
212 acid phosphatase were measured fluorometrically using MUB (methylumbelliferone)-linked  
213 model substrates (DeForest, 2009). While for aminopeptidase and catalase,  
214 L-Leucine-7-amino-4-methylcoumarin and L-DOPA (3, 4-dihydroxy phenylalanine) were used as  
215 substrates. Model substrates and operation procedure in detail were listed in Table S2. Enzyme

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216 activities were expressed in units of  $\text{nmol h}^{-1} \text{g}^{-1}$  and calculated in the method from DeForest  
217 (2009).

218

### 219 *2.7 Quality assurance and control*

220 For As concentration analysis using HG-AFS, As standards were prepared using As stock solutions  
221 (GBW08611, Chinese Metrology Institute of Science and Technology, Beijing, China). The  
222 correlation coefficients of the obtained linear equation reached 0.9993. A certified reference  
223 material (CRM) water-sample (GBWZ50004-88, Institute for Environmental Reference Materials,  
224 Ministry of Environmental Protection, Beijing, China), elemental spikes, and blanks were  
225 incorporated as part of stringent quality control protocols, which were analysed at the beginning,  
226 after every 10 samples and at the end of each run.. Furthermore, every sample was measured in  
227 triplicate. To verify the digestion procedures, Spinach CRM (GBW10015, Institute of geophysical  
228 and geochemical exploration, Chinese Academy of Geological Sciences) was incorporated into  
229 every sample batch. The accuracy of the As measurement of the spinach CRM ranged from  
230 99-108%. The overall accuracy of the soil As fractionation procedure, as determined by  
231 comparing the sum of As determined in all five fractions with a single total As determination, was  
232 found to be within the range of 86-113%.

233

### 234 *2.8 Statistical analysis*

235 All experimental data was processed with Microsoft Excel 2003 and expressed with mean  $\pm$   
236 standard error (SE). Tukey test was applied to the one-way analysis of variance ( $P < 0.05$ ) with the  
237 use of SPSS 16.0 software (SPSS Inc., Chicago, IL). For in-situ XANES analysis, the relative weight of

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238 each As speciation was obtained via linear combination fitting (LCF) of the XANES spectra with  
239 model compounds using IFEFFIT software. The R-factor representing the goodness-of-fit  
240 parameter was calculated. Good fits occur for  $R < 0.05$ . In this study, R-factor was below than  
241 0.01 at each fitting.

242

### 243 **3. Results**

#### 244 *3.1 Biomass and As contents of water spinach grown in soils after inoculation*

245 Inoculating with chlamydo spores of *T. asperellum* SM-12F1 significantly improved the biomass of  
246 water spinach (Table 1). For CZ soils, when inoculation level reached 5%, the height, shoot dry  
247 weight, and root dry weight of water spinach significantly ( $P < 0.05$ ) increased by 35%, 216%, and  
248 87%, respectively, compared with the control. For SM soils, compared with control, the height,  
249 shoot dry weight, and root dry weight of water spinach significantly ( $P < 0.05$ ) increased by 64%,  
250 141%, and 148%, respectively, when inoculation level reached 5%. When both soils at equivalent  
251 inoculation levels were compared, water spinach biomass in SM soils was significantly ( $P < 0.05$ )  
252 higher than in CZ soils.

253

254 Inoculating with chlamydo spores of *T. asperellum* SM-12F1 at different soil loadings affected both  
255 As uptake and assimilation by water spinach, a trend observed in both soils (Table 2). For CZ soils,  
256 when inoculation level reached 5%, As concentration ( $7.75 \text{ mg kg}^{-1}$ ) and contents ( $19.92 \text{ } \mu\text{g pot}^{-1}$ )  
257 in the shoot of water spinach significantly ( $P < 0.05$ ) increased by 139.2% and 637.8% compared  
258 with their control. While no significant change was observed for As concentration and content in  
259 root. For SM soils, when inoculation level reached 5%, the As concentration ( $98.83 \text{ mg kg}^{-1}$ ) in

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260 root significantly ( $P < 0.05$ ) decreased by 15% compared with the control (115.99 mg kg<sup>-1</sup>).  
261 However, the As content (178.60 µg pot<sup>-1</sup>) in root significantly ( $P < 0.05$ ) increased by 100.6%  
262 compared with the control (89.05 µg pot<sup>-1</sup>), due to the higher dry weight of root. No significant  
263 change for As concentration and content in shoot was observed after inoculation. Furthermore,  
264 for BCF and IF of As, no significant difference was found in SM soils after inoculation. However,  
265 the BCF and IF in CZ soils both significantly ( $P < 0.05$ ) increased by 150% compared with the  
266 control, when chlamydo spores were inoculated at 5%. Inoculating with chlamydo spores at 5%  
267 into CZ soils considerably promote the As translocation in water spinach.

268

### 269 3.2 Fungal augmentation and soil available As content after inoculation

270 The successful augmentation of *T. asperellum* SM-12F1 in both soils was observed after  
271 inoculation with chlamydo spores (Fig S1 in the Supplementary Data). For both soils without  
272 inoculation, the survival number of *T. asperellum* SM-12F1 was about 47.5-60.0 cfu g<sup>-1</sup> fresh soils.  
273 When chlamydo spore was inoculated at 1%, the survival numbers of *T. asperellum* SM-12F1  
274 reached 1.4 ×10<sup>4</sup> and 7.4×10<sup>6</sup> cfu in fresh CZ and SM soils, respectively. When inoculation level of  
275 chlamydo spores increased to 5%, the survival numbers of *T. asperellum* SM-12F1 were 1.6 ×10<sup>5</sup>  
276 and 5.0×10<sup>8</sup> cfu in fresh CZ and SM soils, respectively. The augmentation of *T. asperellum*  
277 SM-12F1 in SM soils was more effective than that in CZ soils.

278

279 Inoculating with chlamydo spores of *T. asperellum* SM-12F1 differentially changed the contents of  
280 available As in both soils (Fig 1). When inoculation level reached 1% and 5%, the contents of  
281 available As were 6.5 and 6.6 mg kg<sup>-1</sup> in CZ soils, respectively, which significantly ( $P < 0.05$ )

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282 increased by 14.1% and 16.0% compared with that in control (5.7 mg kg<sup>-1</sup>). For SM soils, however,  
283 no significant change was found for available As when inoculation level was 1%. While the  
284 content of available As (4.5 mg kg<sup>-1</sup>) significantly ( $P < 0.05$ ) decreased by 13% compared with that  
285 in control (5.2 mg kg<sup>-1</sup>).

286

### 287 3.3 Soil pH and As fractionations after inoculation

288 Inoculation with chlamyospores was responsible for a decreasing in soil pH (Fig S2). For both  
289 soils, no significant change in soil pH was found after inoculation with chlamyospores at 1%.  
290 However, when inoculation level reached 5%, the pH values in CZ and SM soils decreased to 6.6  
291 and 5.0 from 7.3 and 5.6 in their corresponding controls, respectively. Comparatively, the pH  
292 values in SM soils were lower than in the CZ soils. This could be explained by the lower original  
293 pH in SM soils and better survival of *T. asperellum* SM-12F1, which are know to release organic  
294 acids.

295

296 Inoculating with chlamyospores of *T. asperellum* SM-12F1 changed the As fractionation  
297 patterns of the soils (Table S3). For both experimental soils, F3 and F2 fractions dominated,  
298 which accounted for approximate 35% and 26% of the total, respectively. Furthermore, with  
299 increasing inoculation level, no significant change was found in the As concentration of the F4  
300 and F5 fractions in CZ soils. When inoculation level of chlamyospores reached 5%, As content in  
301 F1 (1.07 mg kg<sup>-1</sup>) significantly ( $P < 0.05$ ) increased by 98% while no significant change was found  
302 in As contents in F2 or F3, compared with their corresponding controls. For SM soils, however, no  
303 significant variance was observed for As content in F3, F4, or F5 among different inoculation

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304 levels. When inoculation level of chlamydo spores reached 5%, As content in F1 (1.47 mg kg<sup>-1</sup>)  
305 significantly ( $P < 0.05$ ) increased by 72.9% while that in F2 (11.4 mg kg<sup>-1</sup>) significantly ( $P < 0.05$ )  
306 decreased by 61.4%, compared with their corresponding controls. Changes in the contents of  
307 non-specifically (F1) and specifically (F2) sorbed As in both soils after inoculation might be  
308 responsible for the variances in available As.

309

#### 310 *3.4 As speciation in soils after inoculation measured using in situ XANES*

311 Inoculating with chlamydo spores of *T. asperillum* SM-12F1 significantly changed the As  
312 speciation and promoted As methylation in both soils. The XANES spectra corresponding to each  
313 soil sample and the spectra for standards of As(III), As(V), MMA, and DMA are presented in Fig S3.  
314 Evaluation of the XANES spectra beyond the absorption edge shows differences in the region of  
315 11,865-11,875 eV among three fungal strains. The relative weight of each As speciation was  
316 obtained via LCF of the XANES spectra with model compounds (Fig 2). For both soils without  
317 inoculation, the dominant species was As(V) with trace amounts of As(III) present, which  
318 accounted for 84-86% and 14-15% of the total, respectively. For CZ soils, after inoculating with  
319 chlamydo spores of 1%, As(III), As(V), and MMA accounted for 46%, 46%, and 8% of the total,  
320 respectively. When inoculation level reached 5%, As(III), As(V), and MMA accounted for 44%,  
321 37%, and 20.0% of the total, respectively. Comparing the As speciation among different  
322 treatments, the relative weight of As(III) significantly ( $P < 0.05$ ) increased after inoculation.  
323 Correspondingly, the relative weight of As(V) significantly ( $P < 0.05$ ) decreased and MMA  
324 emerged. For SM soils, however, after inoculating with chlamydo spores at 1%, As(III), As(V),  
325 MMA, and DMA accounted for 26%, 54%, 17%, and 3% of the total, respectively. When



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326 inoculation level reached 5%, As(III), As(V), and MMA accounted for 19%, 64%, and 17% of the  
327 total, respectively. Comparing the As speciation trends among different treatments, no  
328 significant change was observed for As(III) or As(V). While organic As species, of MMA and DMA  
329 emerged after inoculation, indicating that microbial inoculation was enhancing As methylation in  
330 both soils.

331

### 332 *3.5 Soil enzymes after inoculation measured using microplate fluorimetry*

333 Inoculating with chlamyospores of *T. asperellum* SM-12F1 significantly and differentially  
334 changed the enzyme activities in two types of soils (Fig 3). For CZ soils, chlamyospores  
335 inoculation lowered the activities of enzymes to different extents. When inoculation level  
336 reached 5%, the activities of  $\beta$ -glucosidase ( $43 \text{ mmol g}^{-1} \text{ h}^{-1}$ ), phosphatase ( $463 \text{ mmol g}^{-1} \text{ h}^{-1}$ ),  
337 aminopeptidase ( $57 \text{ mmol g}^{-1} \text{ h}^{-1}$ ), and catalase ( $298 \text{ mmol g}^{-1} \text{ h}^{-1}$ ) significantly ( $P < 0.05$ )  
338 decreased by 81%, 54%, 60%, and 67% compared with these in controls, respectively. While no  
339 significant change was found for chitinase activities. For SM soils, when inoculation level of  
340 chlamyospores reached 5%, the activities of  $\beta$ -glucosidase ( $3163 \text{ mmol g}^{-1} \text{ h}^{-1}$ ), chitinase ( $5700$   
341  $\text{mmol g}^{-1} \text{ h}^{-1}$ ), and phosphatase ( $3100 \text{ mmol g}^{-1} \text{ h}^{-1}$ ) significantly ( $P < 0.05$ ) improved by 155%,  
342 211%, and 108% compared with these in the controls, respectively. While no significant change  
343 was found for the activities of catalase and aminopeptidase activity significantly decreased after  
344 inoculation.

345

## 346 **4. Discussion**

### 347 *4.1 Inoculating with chlamyospores of T. asperellum SM-12F1 promotes the growth of water*

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348 *spinach*

349 *Trichoderma* has been extensively exploited in agriculture for plant growth promotion, biological  
350 control, modification of plant metabolism (Hoyos-Carvajal et al., 2009), environmental  
351 bioremediation (Wang et al., 2015). *T. asperellum* exhibited some plant growth-promoting  
352 attributes of phosphate solubilization, ACC deaminase activity, auxin, and siderophore  
353 production (Qi and Zhao, 2013). This might be responsible for the growth improvement of water  
354 spinach (Table 1). Furthermore, the activities of phosphatase, chitinase, and  $\beta$ -glucosidase in SM  
355 soils were improved considerably when 5% of chlamydo spores inoculated (Fig 3). This might  
356 contribute to the growth of water spinach. For CZ soils, however, significant decreases in enzyme  
357 activities except for chitinase were found after inoculation. Further analysis showed that the  
358 activities of  $\beta$ -glucosidase ( $R^2=0.6309$ ,  $n=12$ ,  $P < 0.01$ ), phosphatase ( $R^2=0.5738$ ,  $n=12$ ,  $P < 0.01$ ),  
359 aminopeptidase ( $R^2=0.3694$ ,  $n=12$ ,  $P < 0.05$ ), and catalase ( $R^2=0.3336$ ,  $n=12$ ,  $P < 0.05$ )  
360 significantly and negatively correlated with the available As contents in CZ soils. The increased  
361 labile As in soils could inhibit the activities of soil enzymes (Bhattacharyya et al., 2008; Liang et al.,  
362 2014).

363

364 Water spinach growth also might be influenced by As phytotoxicity. In this case, significant  
365 increase in As contents of water spinach shoot in CZ soils was observed and while no significant  
366 change was found in SM soils after inoculation (Table 2). However, the water spinach biomass  
367 greatly increased in both soils. Arsenic speciation in biomass might be changed after inoculation,  
368 which sequentially lowered the phytotoxicity of As. This is supported by the observation that  
369 inoculation resulted in the emergence of organic As species in the soil, which is regarded as a

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370 detoxification process (Fig 2). Tripathi et al. (2015) suggested that As methylation occurred in soils  
371 after inoculating *Trichoderma* could alleviate As stress in chickpea. The result from Cattani et al.  
372 (2015) showed that inoculating *Rhizophagus irregularis* changes As speciation and toxicity in  
373 maize shoot but didn't alter total As concentrations. Based on the above mentioned, in our  
374 opinion, the plant growth might be determined by the synergetic effects of the plant  
375 growth-promoting traits of *T. asperellum* and the changes in soil available As contents, soil  
376 enzyme activities, and As phytotoxicity.

377

#### 378 4.2 Inoculation with chlamydospores of *T. asperellum* SM-12F1 changes As availability in soils

379 The changes of soil pH, As fractionations, and chemical valence can greatly influence As  
380 availability in soils (Quazi et al., 2011). In this study, available As contents significantly increased  
381 in CZ soils while they decreased in SM soils after inoculation (Fig 1). Linear regression analysis  
382 indicated that there was a significantly positive relationship ( $Y=0.545X+1.9067$ ,  $R^2=0.41$ ,  $P < 0.01$ )  
383 between the available As contents in SM soils (Y) and soil pH (X). The lower pH might favor the  
384 decrease of As availability in soils via ligand exchange reactions or electrostatic interactions with  
385 soil minerals (Dixit and Hering, 2003). For CZ soils, however, it was difficult to explain the changes  
386 of As availability based on soil pH.

387

388 As fractionations in soils can greatly affect As availability. In order to better explain the  
389 relationship between As fractionations (X) and available As contents (Y), a stepwise regression  
390 equation was applied to the CZ soils: Available As= $0.068+2.187F1+0.076F3$  ( $R^2=0.9506$ ,  $P < 0.01$ ).

391 Comparatively, As in F1 was the dominant factor for As availability change, due to its higher

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392 coefficient than F3 (0.076). This means that the increase of non-specially absorbed As was  
393 responsible for the augment of available As contents in CZ soils. For SM soils, a stepwise  
394 regression equation could also be derived: Available As=4.135+0.023F2 ( $R^2=0.7191$ ,  $P < 0.01$ ).  
395 This relationship indicates that the significant decrease in available As in SM soils was due to  
396 changes to the pool of As associated with amorphous and poorly-crystalline hydrous oxides of Fe  
397 and Al.

398

399 Arsenic speciation transformation can change As availability in soils. After inoculation, the  
400 relative weight of As(III) in CZ soils was significantly higher than that without inoculation (Fig 2).  
401 This might be helpful in explaining the increased availability As in CZ soils. Because As(III) is not  
402 absorbed as strongly to soil as As(V), and hence has a greater mobility (Chatain et al., 2005). For  
403 SM soils, however, no significant change was observed for in the proportion of As(III) or As(V)  
404 among treatments. Importantly, *T. asperellum* SM-12F1 inoculation caused As methylation in  
405 both soils (Fig 2). This was consistent with the results of Tripathi et al. (2015), who found that  
406 MMA and DMA contents increased in rhizosphere soils of chickpea after being inoculated with  
407 *Trichoderma*. Organic As is seemed with less toxicity and mobility than inorganic As (Akter et al.,  
408 2005). Methylation will lower the toxic As stress to water spinach growth and change As  
409 availability in soils. In this study, XANES method is used to determine As speciation in soil  
410 samples rather than the typical method. Because XANES with the advantages of requiring no  
411 sample preparation and chromatographic separation, has been certified to be a valuable and  
412 reliable tool to detect As speciation (Su et al. 2015; Zeng et al. 2015).

413

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414 4.3 Future application of *T. asperellum* SM-12F1 chlamydo spores in remediation of  
415 As-contaminated soils

416 *T. asperellum* SM-12F1 inoculation showed differential effects on As uptake and transfer in water  
417 spinach between two types of soil. For CZ soils, the water spinach biomass, As content in shoot,  
418 BCF, and TF significantly improved after inoculation (Table 2). It might be feasible to improve the  
419 bioremediation efficiency by inoculating *T. asperellum* SM-12F1 in CZ soils. The results from  
420 Lampis et al. (2015) indicated that inoculation with growth-promoting rhizobacteria increased  
421 biomass of hyperaccumulator *Pteris vittata* by up to 45% and increased As removal efficiency  
422 from 13% without bacteria to 35%. For SM soils, however, water spinach biomass significantly  
423 increased while no significant change in As contents of shoot, BCF, and TF after inoculation was  
424 observed (Table 2). It is recommended to inoculate *T. asperellum* SM-12F1 into SM soils where  
425 planted with crops with the lower ability to uptake As or bioenergy crops such as maize or  
426 sugarcane. The results from Cattani et al. (2015) showed that inoculation with *Rhizophagus*  
427 *irregularis* in combination with phosphorus application could augment the maize biomass but  
428 make no effect on total As content in shoot. Babu et al. (2014a) suggested that inoculation with *T.*  
429 *virens* PDR-28 is beneficial for heavy metal phytostabilization and maize biomass production as a  
430 potential source of bio-fuel in the quest for renewable energy.

431

432 **5. Conclusions**

433 *T. asperellum* SM-12F1 inoculation significantly promoted the growth of water spinach. However,  
434 the effects on As uptake and transfer in water spinach and As availability varied between two  
435 types of As-contaminated soils. Inoculation significantly increased the As content and BCF as well

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436 as IF of As in water spinach and As availability in CZ soils, while no significant change for these  
437 items was found in SM soils. These observations will be helpful for the future application of *T.*  
438 *asperellum* SM-12F1 chlamydo spores in the bioremediation of different As-contaminated soils.

439

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619 **Table 1** Heights and biomass weights of water spinach grown in As-contaminated Chenzhou (CZ)  
620 and Shimen (SM) soils inoculated with different levels of chlamydo spores of *T. asperellum*  
621 SM-12F1.

Treatments	<i>Water spinach</i>		
	Height cm	Shoot dry weight g pot <sup>-1</sup>	Root dry weight g pot <sup>-1</sup>
CZ soils			
Control	12.2±0.6 b	0.8±0.1 c	0.6±0.1 b
1%	14.6±0.9 b	1.5±0.2 b	0.7±0.1 ab
5%	16.4±0.7 a	2.6±0.2 a	1.1±0.1 a
SM soils			
Control	25.2±0.7 b	2.5±0.4 b	0.8±0.1 b
1%	41.4±2.0 a	4.6±0.7 a	1.1±0.2 b
5%	41.1±1.0 a	5.9±0.3 a	1.7±0.2 a

622 The different lowercase letter indicates significant difference ( $P < 0.05$ ) in heights or biomass  
623 weights among different inoculation levels in an individual soil. Data is shown as average value ±  
624 standard error (SE).

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625 **Table 2** Arsenic content and bioconcentration factors (BCF) as well as translocation factors (TF) of  
 626 As in water spinach grown in As-contaminated Chenzhou (CZ) and Shimen (SM) soils inoculated  
 627 with different levels of chlamydo spores of *T. asperellum* SM-12F1.

Treatments	As in shoot of <i>Water spinach</i>		As in root of <i>Water spinach</i>		BCF	TF
	mg kg <sup>-1</sup>	μg pot <sup>-1</sup>	mg kg <sup>-1</sup>	μg pot <sup>-1</sup>		
<b>CZ soils</b>						
Control	3.2±0.5 b	2.7±0.6 b	52.7±4.7 a	32.3±9.2 a	0.02±0.00 b	0.06±0.01 b
1%	4.4±1.0 b	7.0±2.0 b	55.9±6.5 a	38.7±7.2 a	0.03±0.01 b	0.08±0.02 b
5%	7.7±0.7 a	19.9±2.6 a	52.9±1.6 a	59.8±9.0 a	0.05±0.01 a	0.15±0.01 a
<b>SM soils</b>						
Control	2.4±0.4 a	6.2±1.7 a	116.0±18.0 a	89.1±18.4 b	0.02±0.00 a	0.02±0.00 a
1%	2.6±0.2 a	12.0±2.5 a	127.0±14.0 a	128.0±16.0 ab	0.02±0.00 a	0.02±0.00 a
5%	2.6±0.4 a	12.8±2.3 a	98.8±8.9 b	178.6±14.3 a	0.01±0.00 a	0.02±0.00 a

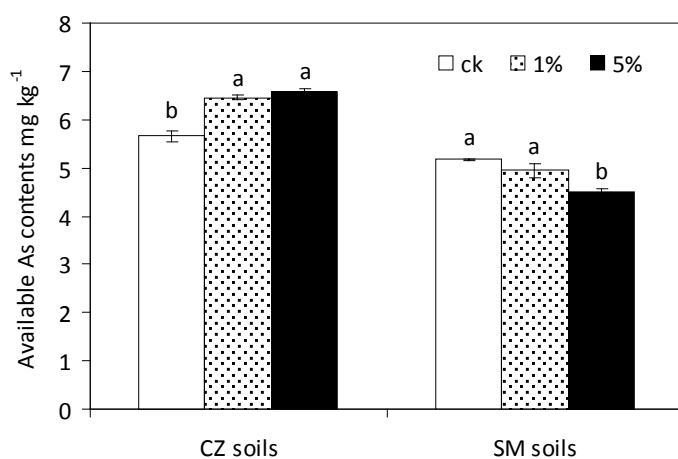
628 The different lowercase letter indicates significant difference ( $P < 0.05$ ) in As concentrations (mg  
 629 kg<sup>-1</sup>), contents (μg pot<sup>-1</sup>), BDF, or IF among different inoculation levels in an individual soil. Data is  
 630 shown as average value ± standard error (SE).

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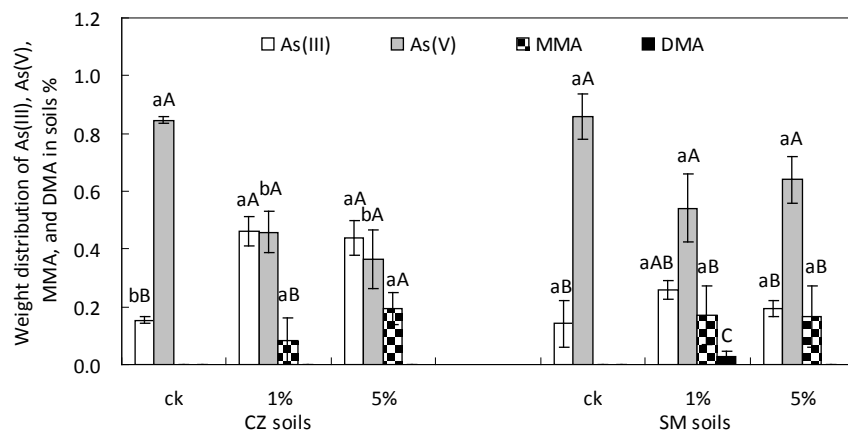
634 **Fig 1.**



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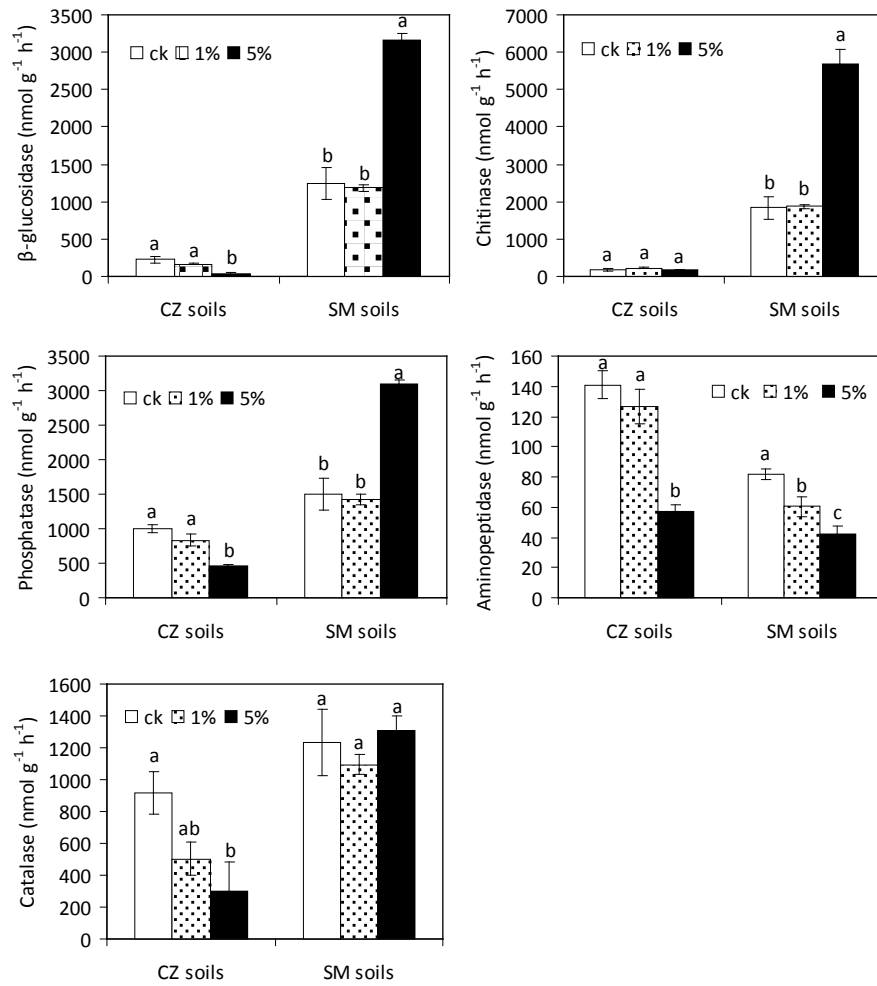
637 **Fig 2.**



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640 **Fig 3.**



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