Selenium uptake, toxicity and reduction in *Flammulina velutipes* supplied with selenite

Jipeng Wang, Bo Wang, Dan Zhang, Yanhong Wu

Recently, selenium (Se) enriched mushrooms have been exploited as diary Se supplements, but our knowledge of the metabolism process during the Se enrichment process is far from complete. In this study, the uptake, toxicity and reduction of selenite in a widely cultivated mushroom, *Flammulina velutipes*, was investigated. The results showed that pH variation (from 5.5 to 7.5), metabolic inhibitor (0.1 mM 2,4-DNP) and P or S starvation led to 11%-26% decreases in the selenite uptake rate of *F. velutipes*. This indicates that a minor portion of the selenite uptake was metabolism dependent, whereas a carrier-facilitated passive transport may be crucial. Growth inhibition of *F. velutipes* initiated at 0.1 mM selenite (11% decrease in the growth rate) and complete growth inhibition occurred at 3 mM selenite. A selenite concentration of 0.03-0.1 mM was recommended to maintain the balance between mycelium production and Se enrichment. *F. velutipes* was capable of reducing selenite to Se(0) nanoparticles, possibly as a detoxification mechanism. This process depended on both selenite concentration and metabolism activity. Overall, the data obtained provided some basic information for the cultivation of the selenized *F. velutipes*, and highlighted the opportunity of using mushrooms for the production of Se(0) nanoparticles.

Selenium uptake, tolerance and reduction in *Flammulina velutipes* supplied with selenite

Jipeng Wang[1,2], Bo Wang[3], Dan Zhang[1], Yanhong Wu[1]

[1]Key Laboratory of Mountain Surface Process and Ecological Regulation, Institute of Mountain Hazards and Environment, Chinese Academy of Sciences (CAS), Chengdu, China

[2]University of Chinese Academy of Sciences, CAS, Beijing, China

[3]Soil and Fertilizer Institute, Sichuan Academy of Agricultural Sciences, Chengdu, China

Yanhong Wu (corresponding author)

#9, Block 4, Renminnanlu Road, Chengdu, Sichuan, 610041, China.

E-mail address: yhwu@imde.ac.cn

Abstract: Recently, selenium (Se) enriched mushrooms have been exploited as diary Se supplements, but our knowledge of the metabolism process during the Se enrichment process is far from complete. In this study, the uptake, tolerance and reduction of selenite in a widely cultivated mushroom, *Flammulina velutipes*, was investigated. The results showed that pH variation (from 5.5 to 7.5), metabolic inhibitor (0.1 mM 2,4-DNP) and P or S starvation led to 11%-26% decreases in the selenite uptake rate of *F. velutipes*. This indicates that a minor portion of the selenite uptake was metabolism dependent, whereas a carrier-facilitated passive transport may be crucial. Growth inhibition of *F. velutipes* initiated at 0.1 mM selenite (11% decrease in the growth rate) and complete growth inhibition occurred at 3 mM selenite. A selenite concentration of 0.03-0.1 mM was recommended to maintain the balance between mycelium production and Se enrichment. *F. velutipes* was capable of reducing selenite to elemental Se [Se(0)] including Se(0) nanoparticles, possibly as a detoxification mechanism. This process depended on both selenite concentration and metabolism activity. Overall, the data obtained provided some basic information for the cultivation of the selenized *F. velutipes*, and highlighted the opportunity of using mushrooms for the production of Se(0) nanoparticles.

Selenium uptake, tolerance and reduction in *Flammulina velutipes* supplied with selenite

Abstract: Recently, selenium (Se) enriched mushrooms have been exploited as diary Se supplements, but our knowledge of the metabolism process during the Se enrichment process is far from complete. In this study, the uptake, tolerance and reduction of selenite in a widely cultivated mushroom, *Flammulina velutipes*, was investigated. The results showed that pH variation (from 5.5 to 7.5), metabolic inhibitor (0.1 mM 2,4-DNP) and P or S starvation led to 11%-26% decreases in the selenite uptake rate of *F. velutipes*. This indicates that a minor portion of the selenite uptake was metabolism dependent, whereas a carrier-facilitated passive transport may be crucial. Growth inhibition of *F. velutipes* initiated at 0.1 mM selenite (11% decrease in the growth rate) and complete growth inhibition occurred at 3 mM selenite. A selenite concentration of 0.03-0.1 mM was recommended to maintain the balance between mycelium production and Se enrichment. *F. velutipes* was capable of reducing selenite to elemental Se [Se(0)] including Se(0) nanoparticles, possibly as a detoxification mechanism. This process depended on both selenite concentration and metabolism activity. Overall, the data obtained provided some basic information for the cultivation of the selenized *F. velutipes*, and highlighted the opportunity of using mushrooms for the production of Se(0) nanoparticles.

Introduction

44 Selenium (Se) is an essential trace element for humans and animals. Supplementation of Se can

45 be necessary, because nutritional Se deficiency affects 500-1000 million people worldwide,

46 especially those from the Keshan disease area of China (Combs 2001). The availability and

47 biological activity of Se depend on its dose and chemical form (Turło et al. 2011). In trace

48 amount, Se confers antioxidant capacities to a number of selenoproteins (Izquierdo et al. 2010).

49 At higher concentrations, Se is toxic because it generates oxidative stress and is involved in

50 DNA damage (Izquierdo et al. 2010; Mániková et al. 2010). Organic Se-containing compounds

51 may exhibit anticarcinogenic activity, and their bioavailability to human beings and animals are

52 considered to be high. (Chun et al. 2006; Suhajda et al. 2000). Recently, there has been

53 increasing interest in using selenized mushrooms as a source of Se supplementation (Egressy-

54 Molnár et al. 2016; Maseko et al. 2013; Rzymski et al. 2016). Mushrooms have long been

55 appreciated for their flavor and texture, as a nutritious food, and as a source of biologically active

56 compounds (Cheung et al. 2003; Yan & Chang 2012). Although the majority of mushrooms are

57 Se-poor, a considerable amount of organic Se can accumulate in mushrooms supplied with

58 selenite or other forms of Se (Falandysz 2008). Turło et al. (2011) reported that the mycelium of

59 *Lentinula edodes* cultured in selenite-fortified substrate accumulated Se in organic compounds,

60 and the synergetic effect of Se compounds and active polysaccharides gave it a strong

61 chemopreventive activity.

62   The transport of selenite through the plasma membrane is the first step of selenite metabolism

63 (Gharieb & Gadd 2004), whereas the mechanism of selenite uptake by mushrooms remains

64   unclear. Selenite is present in the culture medium in different forms depending on the pH,

65   including H_2SeO_3, $HSeO_3^-$ and SeO_3^{2-}, which are mediated by different transporters. In algae,

66   both specific and nonspecific transport systems are involved in the selenite uptake process (Araie

67   et al. 2011; Morlon et al. 2006; Obata et al. 2004). The specific one is driven by ΔpH energized

68   by H^+-ATPase (Araie et al. 2011), and both systems can be associated with transporters used by

69   macronutrients such as phosphate, sulfate and nitrate (Araie et al. 2011; Morlon et al. 2006). In

70   higher plants, selenite in the form of H_2SeO_3 is passively transported through aquaporins (Zhang

71   et al. 2006; Zhang et al. 2010; Zhao et al. 2010), while $HSeO_3^-$ is absorbed in a carrier-mediated

72   way, sharing common transporters with phosphate or sulfite (Li et al. 2008; Zhang et al. 2006).

73   In yeasts, selenite is found to be absorbed in a metabolism dependent way, using the transporter

74   of phosphate or monocarboxylate (Gharieb & Gadd 2004; Lazard et al. 2010; McDermott et al.

75   2010). Moreover, Gharieb & Gadd (2004) also reported a fast, metabolism-independent process

76   during the selenite uptake of *Saccharomyces cerevisiae*. Mushrooms may share similar pathways

77   of selenite uptake with these eukaryotic organisms, and thus medium pH, metabolism activity

78   and competitive nutrients may serve as the major factors regulating the selenite uptake in

79   mushrooms.

80   Mycelial growth and fructification of mushrooms can be hampered by toxic amounts of

81   selenite in the growth substrate (Dong et al. 2012; Niedzielski et al. 2015; Nunes et al. 2012;

82   Turło et al. 2010b). Therefore, an understanding of the tolerance of mushrooms to selenite is

83   necessary to maintain the balance between mycelial production and Se enrichment. In addition to

84   the growth changes when exposed to selenite, the mycelium of some mushrooms were reported

85   to turn red due to the formation of Se(0) (Gharieb et al. 1995; Turło et al. 2010a; Vetchinkina et

86   al. 2013; Vetchinkina et al. 2016). Bioreduction of selenite to Se(0) has long been observed

87   (Levine 1925). Some bacteria and archaea grow anaerobically by linking the oxidation of organic

88   substrates or H_2 to the dissimilatory reduction of Se oxyanions (Lovley 1993; Stolz & Oremland

89   1999). Other organisms, including bacteria (Oremland et al. 2004), fungi (Falcone & Nickerson

90   1963; Gharieb et al. 1995; Nickerson & Falcone 1963) and plants (Zhu et al. 2009), can also

91   reduce selenite to Se(0), most likely as a detoxification mechanism. The biologically formed

92   Se(0) may partly exist as nanoparticles (Vetchinkina et al. 2013; Vetchinkina et al. 2016) which

93   have novel biological activities (e.g., serve as an antioxidant, chemopreventive, and

94   chemotherapeutic agent) and low toxicity (Zhang et al. 2012). Turło et al. (2010) suggested that

95   Se(0), most likely in the nano-colloidal form, was responsible for the enhanced antioxidative

96   activity of the mycelial extracts of the selenized *L. edodes*. Therefore, it is worthwhile to test

97   mushrooms for the potentials of Se(0) production and to further examine the factors influencing

98   this process.

99   *Flammulina velutipes* is among the four most widely cultivated mushrooms worldwide due to

100   its desirable taste and nutritional values (Jing et al. 2014; Yang et al. 2016). With the high

101   efficiency of Se accumulation (Lin et al. 1997), *F. velutipes* serves as a potential source of Se

102   supplementation and biotransformation. In this study, we examined the uptake, tolerance and

103   reduction of Se in *F. velutipes* supplied with 0-5 mM of selenite. In addition, the abilities of *F.*

104   *velutipes* in selenite tolerance and reduction were compared with 11 other species of mushrooms.

105   Thus, the relative sensitivity of *F. velutipes* to selenite and the universality of the selenite

106   reduction process among mushroom species can be understood. The objectives of this study were:

107   (1) to reveal the mechanisms of selenite uptake by *F. velutipes*; (2) to find out the optimal

108   concentration of selenite for the cultivation of selenized *F. velutipes*; (3) to test the ability of *F.*

109   *velutipes* to transform selenite to Se(0) and find out its influencing factors.

110   ## Materials & Methods

111   ### Strains and culture conditions

112   The *F. velutipes* strain used in this study was obtained from the Soil and Fertilizer Institute,

113   Sichuan Academy of Agricultural Sciences, China. Stock cultures were maintained on glucose-

114   yeast (GY) agar plate consisting of glucose (20 g/L), yeast extract (5 g/L) and agar (18 g/L) at

115   4°C in dark. Inoculum of 7 mm in diameter was picked up from the stock culture and inoculated

116   onto the center of a GY agar plate (90 mm in diameter). After cultivated at 25°C in dark until 2/3

117   of the plate was covered by fungal colony, the marginal parts of the fungal colony were used as

118   sources of inocula for selenite uptake, tolerance and reduction experiments. For the other 11

119   species of mushrooms (Table S1 and S2), the same culture conditions were used as *F. velutipes*.

120   ### Selenite uptake experiments

121   Mycelial pellets for the uptake experiments were obtained through shaking cultivation in the GY

122   medium for 12 days (2 inocula of 7 mm in diameter). The composition of the nutrient solution

123   used for selenite uptake (uptake solution) was 15 g/L glucose, 3 g/L arginine, 1.37 g/L KCl, 0.5

124   g/L $MgCl_2 \cdot 6H_2O$. The pH of the solution was buffered at 6.0 with 2 mM MES (2-

125   morpholinoethanesulphonic acid, pH adjusted with NaOH). After the uptake process, a

126   desorption solution was used to get rid of the selenite adhering on the surface of the mycelial

127 pellets. The composition of the desorption solution was 0.136 g/L KH_2PO_4, 0.172 g/L

128 $CaSO_4 \cdot 2H_2O$. The solution was buffered at pH 6.0 with 2 mM MES and stored at 4°C. To test

129 the influence of pH on selenite uptake, the pH of the uptake solution was buffered at 5.5 and 6.5

130 with 2 mM MES, and at 7.5 with 2 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic

131 acid, pH adjusted with HCl). To test if the transporters of phosphate or sulfite were involved in

132 selenite uptake, a P or S starvation treatment was conducted in a pretreatment solution before the

133 uptake process. The composition of the pretreatment solution was 15 g/L glucose, 3 g/L arginine,

134 1.5 g/L K_2HPO_4 0.6 g/L $MgSO_4 \cdot 7H_2O$ for the control treatment. The pH of the solution was

135 buffered at 6.0 with 2 mM MES. In the –P or –S treatment, the K_2HPO_4 or $MgSO4 \cdot 7H2O$ were

136 replaced by the corresponding chloride salts.

137 After shaking cultivation for 12 days, 30 mycelial pellets (5-10 mm in diameter) were gently

138 picked up with tweezers, washed with 100 mL sterile water and 100 mL uptake solution, and

139 then transferred into a 250 mL Erlenmeyer flask containing 100 mL uptake solution. After an

140 adaption process of 30 min, 1 mL 1 mM Na_2SeO_3 (sterilized with a 0.2 μm filter) was added to

141 the Erlenmeyer flask to obtain a final selenite concentration of 0.01 mM and start the uptake

142 process. The flask was then stoppered and shaken at 120 rpm and 25°C for 60 min. The uptake

143 process was stopped by transferring the mycelial pellets to the desorption solution (4°C). After

144 desorption for 15 min, the mycelial pellets were sopped up with filter paper and dried at 50°C.

145 For the 2,4-DNP (2,4-Dinitrophenol) treatment, 100 μL 0.1 M 2,4-DNP dissolved in ethanol was

146 added to the uptake solution (a final 2,4-DNP concentration of 0.1 mM) after the adaption

147 process. Thirty minutes later, the uptake process was started as stated above. An additional

148 control treatment of 0.1% (v/v) ethanol was included. For the –P or –S treatment, the mycelial

149 pellets were cultivated in the pretreatment solution for 24 h before transferring into the uptake

150 solution. A control treatment without P or S starvation was included. All the uptake experiments

151 were performed under sterile conditions. Four replicates were conducted for each treatment.

152 The oven-dried mycelial pellets were grounded in an agate mortar. A subsample of ~0.1 g was

153 weighted with an analytical balance (± 0.0001 g) and digested with 8 mL 68%-70% HNO_3 in a

154 microwave oven (CEM Mars 6, CEM, USA). A standard reference material (Full name: CRM

155 Citrus Leaf, GBW 10020; Se concentration: 0.17 ± 0.03 mg Se/kg; produced by: Institute of

156 Geophysical and Geochemical Exploration, Chinese Academy of Geological Sciences, China)

157 and blank samples were digested together with the mycelial pellets. The Se concentration in the

158 solution was determined using an inductively coupled plasma-mass spectrometry (ICP-MS,

159 NexION 300X, Perkin Elmer, USA).

160 **Selenite tolerance and reduction experiments**

161 The cultivation of *F. velutipes* was carried out in the GY solid and liquid media supplemented

162 with 0-5 mM Na_2SeO_3. Selenite was added to the culture medium at 50-55°C (solid medium) or

163 room temperature (liquid medium) from a stock solution (1 M) after sterilized with a 0.2 μm

164 filter.

165 The tolerance experiment was conducted in solid and liquid media with initial selenite

166 concentrations of 0-5 mM. The 9 selenite concentrations tested were 0, 0.001, 0.01, 0.03, 0.1, 0.3,

167 1, 3 and 5 mM. For solid cultivation, an isolate (7 mm in diameter) was inoculated onto the

168 center of test plate containing 0-5 mM selenite, and incubated at 25°C in the dark. The static

169   cultivation was conducted in a 50 mL flask containing 20 mL of medium and was inoculated and

170   cultivated in the same way as the solid cultivation. The biomass of the 20-day-old mycelia was

171   determined after oven-dried at 60°C. The shaking cultivation was conducted in a 250 mL flask

172   containing 100 mL of medium with 2 inocula. After cultivation at 25°C and 120 rpm in dark for

173   13 days, the biomass of the mycelial pellets was determined after oven-dried at 60°C. The radical

174   growth rate, density, height, pigment secretion and other colony characteristics were recorded

175   daily. Each treatment was performed with 4 replicates for the shaking cultivation and with 5

176   replicates for the solid and static cultivations. For the other 11 species of mushrooms, the

177   tolerance experiments were conducted in solid cultivation with a selenite concentration of 0.1

178   mM in the same way as *F. velutipes*.

179   For the reduction experiment, the ability of *F. velutipes* to reduce selenite to Se(0) was

180   determined visually, and the degree of red coloration resulted from Se(0) formation was used as

181   an indication of reduction (Gharieb et al. 1995; no coloration, pink, pale red, and red represented

182   no, weak, moderate, and strong reduction, respectively). During the tolerance experiment with

183   initial selenite concentrations of 0-5 mM, the degree of red coloration of the fungal colonies was

184   recorded daily. In addition, the reduction ability was examined after the full development of the

185   mycelium. For this purpose, *F. velutipes* was first cultivated in selenite-free medium for 14 days

186   until selenite was added to the medium to a final selenite concentration of 0 to 3 mM. Each

187   treatment was performed in triplicate. The coloration of the mycelial pellets was observed hourly

188   for the first 10 h and again at 24 h and 32 h. All of the tolerance and reduction experiments were

189   conducted under sterile conditions. For the other 11 species of mushrooms, the selenite reduction

190   was determined visually in solid cultivation (0.1 mM selenite) and shaking cultivation (0.3 mM

191   selenite).

192   For the TEM (transmission electron microscope) studies, the mycelia were picked from the red

193   region of a 20-day-old colony (static cultivation, 0.3 mM selenite) with an inoculating needle and

194   washed 3 times with a 0.01 M PBS solution (NaCl 8.01 g/L, KCl 0.20 g/L, $Na_2HPO_4 \cdot 12H_2O$

195   3.58 g/L, KH_2PO_4 0.27 g/L; pH 7.4). The mycelia were then suspended in a 1.5 mL centrifuge

196   tube containing 0.5 mL of water and scattered in an ultrasonic cleaner. A drop of the obtained

197   mixture was placed onto the copper grids using a rubber head dropper. After drying the sample, a

198   transmission electron microscope (FEI Tecnai G2F20 S-TWIN, USA) working at 200 kv was

199   used for morphology and energy dispersive X-ray spectroscopy (EDX) analysis.

200   **Statistical analysis**

201   Comparisons among the mycelial biomass in static and shaking cultivations were performed by

202   analysis of variance (ANOVA) followed by Duncan's multiple range test. The mycelial growth

203   after the initial adaption period in the solid cultivation was fitted by a linear regression, and the

204   differences between the slope (k) of the control treatment and the selenite treatment were tested

205   (Table S3). The SAS 9.1 (SAS Institute, Inc., Cary, North Carolina, USA) software package was

206   used for all of the statistical analysis.

207   **Results**

208   **Influences of pH, metabolic inhibitor and nutrient starvation on the selenite uptake**

209   The rate of selenite uptake was not significantly influenced by the medium pH ($p > 0.05$), yet its

210   mean values decreased by 14% as the medium pH increased from 5.5 to 7.5 (Fig. 1A). The

211 addition of 0.1 mM 2,4-DNP significantly inhibited the selenite uptake by 11% ($p < 0.05$) (Fig.

212 1B). The rate of selenite uptake responded similarly to the P and S starvation, and decreased by

213 25% and 26% after the P and S starvation, respectively ($p < 0.05$) (Fig. 1C).

214 **Growth responses of *F. velutipes* to 0-5 mM selenite**

215 The growth rate of *F. velutipes* in the solid cultivation began to be inhibited at 0.1 mM selenite

216 (growth rate decreased by 11%) (Fig. 2A; Table S3). The inhibition increased with the increasing

217 selenite concentration. The mycelial growth stopped after 5 days in the media containing 1 mM

218 selenite (Fig. 2A), and the colony margins became corral-like (Fig. S1). No mycelial growth was

219 observed at the selenite concentrations of 3 mM or higher (Fig. 2A). After different durations of

220 exposures to 3 or 5 mM selenite, the inocula were then re-inoculated to the selenite-free media.

221 Longer lag periods were observed for the selenite stressed inocula, whereas the growth rates after

222 germination were not severely impaired, except when the inocula were exposed to 5 mM selenite

223 for 65 days (Fig. 2B; Table S4). In the static cultivation, the inhibition of mycelial growth started

224 when the selenite concentration reached 0.1 mM, and the inhibition intensified as the selenite

225 concentration increased. No mycelial growth was observed in the media containing selenite

226 concentrations of 3 mM or higher (Fig. 3C). In the shaking cultivation, the responses of mycelial

227 growth were similar to those in the static cultivation, except that significant growth inhibition

228 started at the selenite concentration of 0.3 mM (Fig. 3D).

229 **Reduction of selenite to Se(0) by *F. velutipes* exposed to 0-5 mM of selenite**

230 When *F. velutipes* was cultivated in the media containing 0-5 mM selenite (Fig. 3A, 3B and 3C),

231 the reduction intensities, as indicated by the red coloration, increased with increasing selenite

232 concentrations in the range of 0-0.3 mM. Faint reduction appeared at the selenite concentration

233 of 0.03 mM and the reduction intensity increased until 0.3 mM. The reduction intensity

234 decreased at the selenite concentration of 1 mM. At the selenite concentrations of 3 mM or

235 higher, there was no sign of mycelial growth or selenite reduction. When the mycelial pellets

236 cultivated in the selenite-free media for 14 days were subjected to 0-3 mM selenite, the reduction

237 intensity increased with increasing selenite concentrations (Fig. 3D). In the solid and static

238 cultivation, denser colors were observed in the central part of the colony (around the inocula)

239 compared to the margins (Fig. 3A and 3B; Fig. S2). In the shaking cultivation, denser colors

240 were observed in the large mycelial pellets, especially those developed from the inocula, than in

241 the newly formed small pellets (Fig. 3C and 3D; Fig. S2).

242 **Discussion**

243 **Possible mechanisms of selenite uptake by *F. velutipes***

244 The changes in selenite forms induced by the varying pH did not significantly influence the

245 selenite uptake by *F. velutipes* (Fig. 1A). From pH 5.5 to pH 7.5, the proportion of $HSeO_3^-$

246 decreased markedly from 92.6% to 11.2%, corresponding to the increase of SeO_3^{2-} from 7.3% to

247 88.8% (Table S5; The proportions of different forms were calculated based on the pK_a values of

248 selenous acid which determine the degree of the protonation of selenite under different pH values:

249 $pK_1 = 2.57$, $pK_2 = 6.60$), whereas the selenite uptake rate only decreased by 14%. The lack of

250 sensitivity of selenite uptake to medium pH of this range was commonly reported in organisms

251 including algae (Araie et al. 2011; Morlon et al. 2006) and higher plants (Zhang et al. 2006;

252 Zhang et al. 2010; Zhao et al. 2010). Thus, when the pH of medium is higher than 5, selenite

253 speciation may not be the key regulator of selenite uptake. However, it was evident that in our

254 and other studies (Araie et al. 2011; McDermott et al. 2010; Zhang et al. 2006; Zhang et al. 2010;

255 Zhao et al. 2010), selenite uptake showed a decreasing trend as medium pH increased from 5 to 8.

256 This may originate from the selenite-proton symport according to the findings of McDermott et

257 al. (2010). Therefore, in *F. velutipes*, a small portion of the selenite might be taken up through a

258 proton-coupled manner.

259   A minor fraction of the selenite was absorbed metabolically by *F. velutipes*. During our short-

260 term uptake experiment, 2,4-DNP (the uncoupler of oxidative phosphorylation) inhibited the

261 selenite uptake by 11% (Fig. 1B). In algae and yeast, the ΔpH generated by H^+-ATPase has been

262 considered as the driving force of the active selenite transport (Araie et al. 2011; McDermott et

263 al. 2010). In combination with the responses of selenite uptake to medium pH, the active selenite

264 absorption by *F. velutipes* may be associated with the anion-proton symporters. The inhibition

265 rate observed in this study was lower compared with similar studies using 2,4-DNP or CCCP

266 (carbonyl cyanide m-chlorophenyl hydrazone) as metabolic inhibitors at near neutral pH

267 (inhibition rates ranging from ~25% to 80%) (Gharieb & Gadd 2004; Li et al. 2008; Zhang et al.

268 2010). This highlighted the significance of the passive transport in the selenite uptake by *F.*

269 *velutipes*. Gharieb & Gadd (2004) observed a fast, metabolism-independent phase in the selenite

270 uptake process of *S. cerevisiae*, and this phase was responsible for the majority of the absorbed

271 Se. The authors attributed this phase to abiotic adsorption and simple diffusion (Gharieb & Gadd

272 2004). Nevertheless, it was unlikely that the selenite uptake in our study proceeded via

273 adsorption or simple diffusion, since the negatively charged cell membrane would repel $HSeO_3^-$

274   and the hydrophobic core of lipid bilayer was impermeable to selenite (Araie et al. 2011; Morlon

275   et al. 2006; Wells & Richardson 1985). Moreover, the desorption procedure adopted in this study

276   following the selenite uptake process further precluded the possibility of abiotic adsorption (see

277   Material & Methods). Therefore, a carrier-facilitated passive transport may be crucial to the

278   selenite uptake by *F. velutipes*.

279   Specific selenite transporters have not been identified, and like other trace anions, selenite

280   may share transporters with the major anions such as phosphate (Lazard et al. 2010; Li et al.

281   2008; Riedel & Sanders 1996), sulfite (Zhang et al. 2006) and monocarboxylate (McDermott et

282   al. 2010). We tested the possible roles of phosphate and sulfite transporters in selenite uptake,

283   and assumed that with the up-regulation of phosphate or sulfite transporter after P or S starvation,

284   the rate of selenite uptake would increase (Li et al. 2008). Opposite to our assumption, the uptake

285   rate of selenite decreased significantly by ~25% after P and S starvation (Fig. 1C). One

286   explanation is that two transporters with contrasting selectivity for selenite might exist in *F.

287   velutipes*. In *S. cerevisiae*, which is phylogenetically close to *F. velutipes*, the selenite uptake

288   involves the high and low affinity phosphate transporters at low and high (1 mM) phosphate

289   concentrations, respectively (Lazard et al. 2010). The high affinity transporter strongly selects

290   phosphate over selenite, whereas the low affinity transporter does not discriminate efficiently

291   between the two anions (Lazard et al. 2010). Thus the low affinity transporter can potentially

292   have a higher conductance to selenite than the high affinity transporter (Lazard et al. 2010).

293   Pinson et al. (2004) observed a close relationship between selenite resistance and the expression

294   of the high affinity phosphate transporter in *S. cerevisiae*. If this transport system applies to *F.

295   *velutipes*, the transporter with high phosphate affinity would be up-regulated, whereas the one

296   with low affinity would be down-regulated following P starvation. Meanwhile, the low affinity

297   phosphate transporter would be responsible for the selenite uptake in the control treatment with

298   high phosphate concentration (8.6 mM). Because the high affinity transporter discriminate

299   selenite more efficiently, less selenite is absorbed after P starvation than in the control treatment.

300   Similar sulfite transport system might be responsible for the decrease in selenite uptake after S

301   starvation. Further studies are needed to identify the transporters of selenite in *F. velutipes*.

302   **Sensitivity of *F. velutipes* to selenite**

303   An overdose of selenite interferes with the S and P metabolism (Bryant & Laishley 1988;

304   Daniels 1996; Tomei et al. 1995; Weissman & Trelease 1955) and leads to oxidative stress by

305   reacting with intracellular thiols (Papp et al. 2007). In this study, selenite began to be toxic to *F.*

306   *velutipes* at 0.1 mM (Fig. 2). Based on the colony morphologies, the responses of *F. velutipes* to

307   0.1 mM selenite were moderate among the commonly cultivated mushrooms (Table S1, S2 and

308   S6). Lin et al. (1997) reported that 0.03 mM and 0.12 mM selenite started to inhibit the growth of

309   *F. velutipes* in solid and liquid cultivations (PDA media), respectively. The discrepancies with

310   our results in selenite sensitivity may be attributed to the differences in strains and medium

311   components. The corral-like colony margins of *F. veluptipes* when subjected to 1 mM selenite

312   for more than 5 days were also reported when *Fusarium* sp. was exposed to 5 mM selenite

313   (Gharieb et al. 1995). It remains unclear if this is a common response of fungal mycelia when

314   subjected to toxic amount of selenite. The mycelial growth completely stopped at selenite

315   concentrations of 3 and 5 mM (Fig. 2A, 2C and 2D), but the inocula resuscitated without a

316   severe decrease in the growth rate after transferred to selenite-free medium (Fig. 2B; Table S4).

317   Thus, a high concentration of selenite induced the dormancy of the inocula without severely

318   damaging their vitality. Based on our results, we recommend a selenite concentration between

319   0.03 mM and 0.1 mM to maintain the balance between Se enrichment and mycelial productivity.

320   This is in agreement with the optimal selenite concentration (0.06 mM) proposed by Ma et al.

321   (2012) for the soluble organic Se accumulation during the fermentation of *F. velutipes* mycelia.

322   **Reduction of selenite to Se(0) by *F. velutipes***

323   The alteration of colony color and the TEM analysis of the red part of the mycelia confirmed the

324   existence of Se(0) (partly as nanoparticles) when *F. velutipes* was treated with selenite (Fig. 3

325   and 4). The method of Se(0) determination in this study (based on colony color; Gharieb et al.

326   1995) was semiquantitative, because the filamentous nature of the fungal mycelia made it

327   difficult for the use of the spectrophotometric method (Dhanjal & Cameotra 2010). Further

328   research is needed to improve the accuracy of Se(0) determination during fungal cultivation.

329   Moreover, the TEM image shows Se(0) particles outside the hyphae of *F. velutipes* (Fig. 4A). It

330   remains unclear whether there was an extracellular pathway for selenite reduction or the Se(0)

331   particles inside the hyphae were leaked out during sample preparation. In addition to yeasts

332   (Falcone & Nickerson 1963; Gharieb et al. 1995; Nickerson & Falcone 1963) and molds (Brady

333   et al. 1996; Gharieb et al. 1995; Ramadan et al. 1988), some species of mushrooms, including

334   *Coriolus versicolor* and *Lentinula edodes*, *Ganoderma lucidum*, *Pleurotus ostreatus* and *Grifola*

335   *frondosa* (Gharieb et al. 1995; Vetchinkina et al. 2013; Vetchinkina et al. 2016) have been

336   studied for their abilities to transform selenite to Se(0). In our study, *F. velutipes* and other 11

337   species of phylogenetically and ecologically varied mushrooms were tested, and 10 of them

338   showed the reducing ability (Fig. S3). Thus it is conceivable that mushrooms commonly possess

339   this reducing ability.

340   Selenite reduction by fungi to Se(0) has generally been considered as a detoxification

341   mechanism (Gharieb et al. 1995; Ramadan et al. 1988; Vetchinkina et al. 2013). If the same is

342   true for *F. velutipes*, the reduction intensity would increase as selenite poisoning strengthens. At

343   selenite concentrations below 0.3 mM, the reduction intensities increased with the increasing

344   selenite concentration (Fig. 3A, 3B and 3C). However, at selenite concentrations of 1 mM or

345   higher, when the mycelial growth was severely inhibited (Fig. 2), the reduction intensities

346   decreased (Fig. 3A, 3B and 3C). Lortie et al. (1992) also reported that selenite poisoning at high

347   concentrations caused a decrease in the reduction rate of selenite by *Pesudomonas stutzeri*.

348   Nickerson & Falcone (1963) proposed that the reduction process operated at the expense of the

349   endogenous metabolism. Selenite acts as a prooxidant at high concentrations which undergoes

350   glutathione-mediated reduction to hydrogen selenide (Izquierdo et al. 2010). The depletion of

351   metabolic products, such as glutathione, might be the reason that the Se(0) production decreased

352   at high selenite concentrations, and thus we assume that the reduction of selenite to Se(0) by *F.*

353   *velutipes* may be metabolism dependent. This idea was tested using mycelial pellets from

354   selenite-free media (14-day-old), as they were supposed to be metabolically active compared

355   with those developed in the media initially containing toxic amounts of selenite. When the

356   mycelia were not constrained by the metabolic activity, the red coloration appeared in 24 h and

357   the reduction intensity increased with the increasing selenite concentrations in the range of 0-3

358   mM (Fig. 3D). This may explain why the inoculum part of the colony showed faster and denser

359   coloration when subjected to selenite (Fig. 3; Fig. S2). Thus, the reduction of selenite to Se(0)

360   can be influenced by both the selenite concentration and the metabolism activity of the fungi.

361   In addition to the red coloration, colonies treated with a toxic amount of selenite often

362   exhibited a garlic smell during cultivation of *F. velutipes* (Fig. 3C and 3D). Similar phenomena

363   have been reported in the literature studying the responses of fungi to selenite, and the garlic

364   smell was suspected to originate from volatile Se-containing compounds, possibly

365   dimethylselenide (Brady et al. 1996; Gharieb et al. 1995; Schilling et al. 2011). Direct evidence

366   is needed to confirm the speciation of the volatile compounds. This may suggest that the

367   reduction of selenite to Se(0) was not the only mechanism for *F. velutipes* to cope with the

368   selenite toxicity. The transformation of selenite to volatile Se species which might appear in this

369   study, together with the reduced uptake (Gharieb et al. 1995) and the reduced incorporation of Se

370   into proteins (Ramadan et al. 1988), may also contribute to the tolerance.

371   **Conclusions**

372   At near neutral pH, selenite uptake was slightly affected by selenite species and metabolic

373   inhibitor. A carrier-mediated passive transport might be crucial to the selenite absorption by *F.*

374   *velutipes*. At selenite concentrations of 0.1 mM or higher, the absorbed selenite led to growth

375   inhibition of *F. velutipes*. As a detoxification mechanism, a portion of the selenite was

376   transformed to Se (0) including Se(0) nanoparticles in a metabolic dependent way. These results

377   provided some basic information for the cultivation of the selenized *F. velutipes* and highlighted

378   the opportunity of using mushrooms for the production of Se(0) nanoparticles.

379 **References**

380 Araie H, Sakamoto K, Suzuki I, and Shiraiwa Y. 2011. Characterization of the selenite uptake mechanism in the

381 coccolithophore *Emiliania huxleyi* (Haptophyta). *Plant and Cell Physiology* 52: 2014-1210.

382 Brady JM, Tobin JM, and Gadd GM. 1996. Volatilization of selenite in aqueous medium by a *Penicillium* species.

383 *Mycological Research* 100:955-961.

384 Bryant RD, and Laishley EJ. 1988. Evidence for two transporters of sulfur and selenium oxyanions in *Clostridium*

385 *pasteurianum*. *Canadian Journal of Microbiology* 34:700-703.

386 Cheung L, Cheung PC, and Ooi VE. 2003. Antioxidant activity and total phenolics of edible mushroom extracts.

387 *Food Chemistry* 81:249-255.

388 Chun JY, Nadiminty N, Lee SO, Onate SA, Lou W, and Gao AC. 2006. Mechanisms of selenium down-regulation

389 of androgen receptor signaling in prostate cancer. *Molecular Cancer Therapeutics* 5:913-918.

390 Combs GF. 2001. Selenium in global food systems. *British Journal of Nutrition* 85:517-547.

391 Daniels LA. 1996. Selenium metabolism and bioavailability. *Biological Trace Element Research* 54:185-199.

392 Dhanjal S, and Cameotra SS. 2010. Aerobic biogenesis of selenium nanospheres by *Bacillus cereus* isolated from

393 coalmine soil. *Microbial Cell Factories* 9: 52.

394 Dong JZ, Lei C, Ai XR, and Wang Y. 2012. Selenium enrichment on Cordyceps militaris Link and analysis on its

395 main active components. *Applied Biochemistry and Biotechnology* 166:1215-1224.

396 Egressy-Molnár O, Ouerdane L, Győrfi J, and Dernovics M. 2016. Analogy in selenium enrichment and selenium

397 speciation between selenized yeast *Saccharomyces cerevisiae* and H*ericium erinaceus* (lion's mane

398 mushroom). LWT - Food Science and Technology 68:306-312.

399 Falandysz J. 2008. Selenium in edible mushrooms. *Journal of Environmental Science and Health, Part C:*

400 *Environmental Carcinogenesis and Ecotoxicology Reviews* 26:256-299.

401 Falcone G, and Nickerson WJ. 1963. Reduction of selenite by intact yeast cells and cell-free preparations. *Journal of*

402 *Bacteriology* 85:754-762.

403 Gharieb M, Wilkinson S, and Gadd G. 1995. Reduction of selenium oxyanions by unicellular, polymorphic and

404 filamentous fungi: cellular location of reduced selenium and implications for tolerance. *Journal of*

405 *Industrial Microbiology* 14:300-311.

406 Gharieb MM, and Gadd GM. 2004. The kinetics of ^{75}[Se]-selenite uptake by *Saccharomyces cerevisiae* and the
407   vacuolization response to high concentrations. *Mycological Research* 108:1415-1422.

408 Izquierdo A, Casas C, and Herrero E. 2010. Selenite-induced cell death in *Saccharomyces cerevisiae:* protective role
409   of glutaredoxins. *Microbiology* 156:2608-2620.

410 Jing P, Zhao S-J, Lu M-M, Cai Z, Pang J, and Song L-H. 2014. Multiple-Fingerprint Analysis for Investigating
411   Quality Control of *Flammulina velutipes* Fruiting Body Polysaccharides. *Journal of Agricultural and Food*
412   *Chemistry* 62:12128-12133.

413 Lazard M, Blanquet S, Fisicaro P, Labarraque G, and Plateau P. 2010. Uptake of selenite by *Saccharomyces*
414   *cerevisiae* involves the high and low affinity orthophosphate transporters. *Journal of Biological Chemistry*
415   285:32029-32037.

416 Levine VE. 1925. The reducing properties of microorganisms with special reference to selenium compounds.
417   *Journal of Bacteriology* 10:217.

418 Li HF, McGrath SP, and Zhao FJ. 2008. Selenium uptake, translocation and speciation in wheat supplied with
419   selenate or selenite. *New Phytologist* 178:92-102.

420 Lin L, Xie BF, Shi QG, and Zhang WH. 1997. Submerged fermentation of Se-accumulating *Flammulina velutipes*
421   and its characters. *Edible Fungi of China* 16:36-38. in Chinese with English abstract.

422 Lortie L, Gould W, Rajan S, McCready R, and Cheng K-J. 1992. Reduction of selenate and selenite to elemental
423   selenium by a *Pseudomonas stutzeri* isolate. *Applied and Environmental Microbiology* 58:4042-4044.

424 Lovley DR. 1993. Dissimilatory metal reduction. *Annual Reviews in Microbiology* 47:263-290.

425 Mániková D, Vlasáková D, Loduhová J, Letavayová L, Vigašová D, Krascsenitsová E, Vlčková V, Brozmanová J,
426   and Chovanec M. 2010. Investigations on the role of base excision repair and non-homologous end-joining
427   pathways in sodium selenite-induced toxicity and mutagenicity in *Saccharomyces cerevisiae*. *Mutagenesis*
428   25:155-162.

429 Ma Y, Xiang F, Xiang J, and Yu L. 2012. Optimization of Soluble Organic Selenium Accumulation during
430   Fermentation of *Flammulina velutipes* Mycelia. *Zeitschrift für Naturforschung C* 67:594-602.

431 Maseko T, Callahan DL, Dunshea FR, Doronila A, Kolev SD, and Ng K. 2013. Chemical characterisation and
432   speciation of organic selenium in cultivated selenium-enriched Agaricus bisporus. *Food Chemistry*

433   141:3681-3687.

434   McDermott JR, Rosen BP, and Liu Z. 2010. Jen1p: a high affinity selenite transporter in yeast. *Molecular Biology of*

435   *the Cell* 21:3934-3941.

436   Morlon H, Fortin C, Adam C, and Garnier-Laplace J. 2006. Selenite transport and its inhibition in the unicellular

437   green alga *Chlamydomonas reinhardtii*. *Environmental Toxicology and Chemistry* 25:1408-1417.

438   Nickerson WJ, and Falcone G. 1963. Enzymatic reduction of selenite. *Journal of Bacteriology* 85:763-771.

439   Niedzielski P, Mleczek M, Siwulski M, Rzymski P, Gąsecka M, and Kozak L. 2015. Supplementation of cultivated

440   mushroom species with selenium: bioaccumulation and speciation study. *European Food Research and*

441   *Technology*:1-8.

442   Nunes RGFL, Luz JMRd, Freitas RdB, Higuchi A, Kasuya MCM, and Vanetti MCD. 2012. Selenium

443   Bioaccumulation in Shiitake Mushrooms: A Nutritional Alternative Source of this Element. *Journal of*

444   *Food Science* 77:983-986.

445   Obata T, Araie H, and Shiraiwa Y. 2004. Bioconcentration mechanism of selenium by a coccolithophorid, Emiliania

446   huxleyi. *Plant and Cell Physiology* 45:1434-1441.

447   Oremland RS, Herbel MJ, Blum JS, Langley S, Beveridge TJ, Ajayan PM, Sutto T, Ellis AV, and Curran S. 2004.

448   Structural and spectral features of selenium nanospheres produced by Se-respiring bacteria. *Applied and*

449   *Environmental Microbiology* 70:52-60.

450   Papp LV, Lu J, Holmgren A, and Khanna KK. 2007. From selenium to selenoproteins: synthesis, identity, and their

451   role in human health. *Antioxidants & redox signaling* 9:775-806.

452   Pinson B, Merle M, Franconi J-M, and Daignan-Fornier B. 2004. Low affinity orthophosphate carriers regulate PHO

453   gene expression independently of internal orthophosphate concentration in Saccharomyces cerevisiae.

454   Journal of Biological Chemistry 279:35273-35280.

455   Ramadan SE, Razak A, Youssef Y, and Sedky N. 1988. Selenium metabolism in a strain of *Fusarium*. *Biological*

456   *Trace Element Research* 18:161-170.

457   Riedel GF, and Sanders JG. 1996. The influence of pH and media composition on the uptake of inorganic selenium

458   by *Chlamydomonas reinhardtii*. *Environmental Toxicology and Chemistry* 15:1577-1583.

459   Rzymski P, Mleczek M, Niedzielski P, Siwulski M, and Gąsecka M. 2016. Potential of cultivated *Ganoderma*

lucidum mushrooms for the production of supplements enriched with essential elements. Journal of Food Science 81:C587-C592.

Schilling K, Johnson TM, and Wilcke W. 2011. Isotope Fractionation of selenium during fungal biomethylation by *Alternaria alternata*. Environmental Science & Technology 45:2670-2676.

Stolz JF, and Oremland RS. 1999. Bacterial respiration of arsenic and selenium. *FEMS Microbiology Reviews* 23:615-627.

Suhajda A, Hegoczki J, Janzso B, Pais I, and Vereczkey G. 2000. Preparation of selenium yeasts I. Preparation of selenium-enriched *Saccharomyces cerevisiae*. *Journal of Trace Elements in Medicine and Biology* 14:43-47.

Tomei FA, Barton LL, Lemanski CL, Zocco TG, Fink NH, and Sillerud LO. 1995. Transformation of selenate and selenite to elemental selenium by *Desulfovibrio desulfuricans*. *Journal of Industrial Microbiology* 14:329-336.

Turło J, Gutkowska B, and Herold F. 2010a. Effect of selenium enrichment on antioxidant activities and chemical composition of *Lentinula edodes* (Berk.) Pegl. mycelial extracts. *Food and Chemical Toxicology* 48:1085-1091.

Turło J, Gutkowska B, Herold F, Gajzlerska W, Dawidowski M, Dorociak A, and Zobel A. 2011. Biological Availability and Preliminary Selenium Speciation in Selenium-Enriched Mycelium of *Lentinula edodes* (Berk.). *Food Biotechnology* 25:16-29.

Turło J, Gutkowska B, Herold F, Klimaszewska M, and Suchocki P. 2010b. Optimization of selenium-enriched mycelium of *Lentinula edodes* (Berk.) pegler as a food supplement. *Food Biotechnology* 24:180-196.

Vetchinkina E, Loshchinina E, Kursky V, and Nikitina V. 2013. Reduction of organic and inorganic selenium compounds by the edible medicinal basidiomycete *Lentinula edodes* and the accumulation of elemental selenium nanoparticles in its mycelium. *Journal of Microbiology* 51:829-835.

Vetchinkina E, Loshchinina E, Kurskyi V, and Nikitina V. 2016. Biological synthesis of selenium and germanium nanoparticles by xylotrophic basidiomycetes. *Applied Biochemistry and Microbiology* 52:87-97.

Weissman GS, and Trelease SF. 1955. Influence of sulfur on the toxicity of selenium to *Aspergillus*. *American Journal of Botany*:489-495.

487   Wells J, and Richardson D. 1985. Anion accumulation by the moss *Hylocomium splendens*: uptake and competition

488   studies involving arsenate, selenate, selenite, phosphate, sulphate and sulphite. *New Phytologist* 101:571-

489   583.

490   Yan H, and Chang H. 2012. Antioxidant and Antitumor Activities of Selenium-and Zinc-Enriched Oyster

491   Mushroom in Mice. *Biological Trace Element Research* 150:236-241.

492   Yang W, Yu J, Pei F, Mariga AM, Ma N, Fang Y, and Hu Q. 2016. Effect of hot air drying on volatile compounds

493   of *Flammulina velutipes* detected by HS-SPME–GC–MS and electronic nose. *Food Chemistry* 196:860-866.

494   Zhang L, Li D, and Gao P. 2012. Expulsion of selenium/protein nanoparticles through vesicle-like structures by

495   *Saccharomyces cerevisiae* under microaerophilic environment. *World Journal of Microbiology and*

496   *Biotechnology* 28:3381-3386.

497   Zhang L, Shi W, and Wang X. 2006. Difference in selenite absorption between high-and low-selenium rice cultivars

498   and its mechanism. *Plant and Soil* 282:183-193.

499   Zhang L, Yu F, Shi W, Li Y, and Miao Y. 2010. Physiological characteristics of selenite uptake by maize roots in

500   response to different pH levels. *Journal of Plant Nutrition and Soil Science* 173:417-422.

501   Zhao XQ, Mitani N, Yamaji N, Shen RF, and Ma JF. 2010. Involvement of silicon influx transporter OsNIP2; 1 in

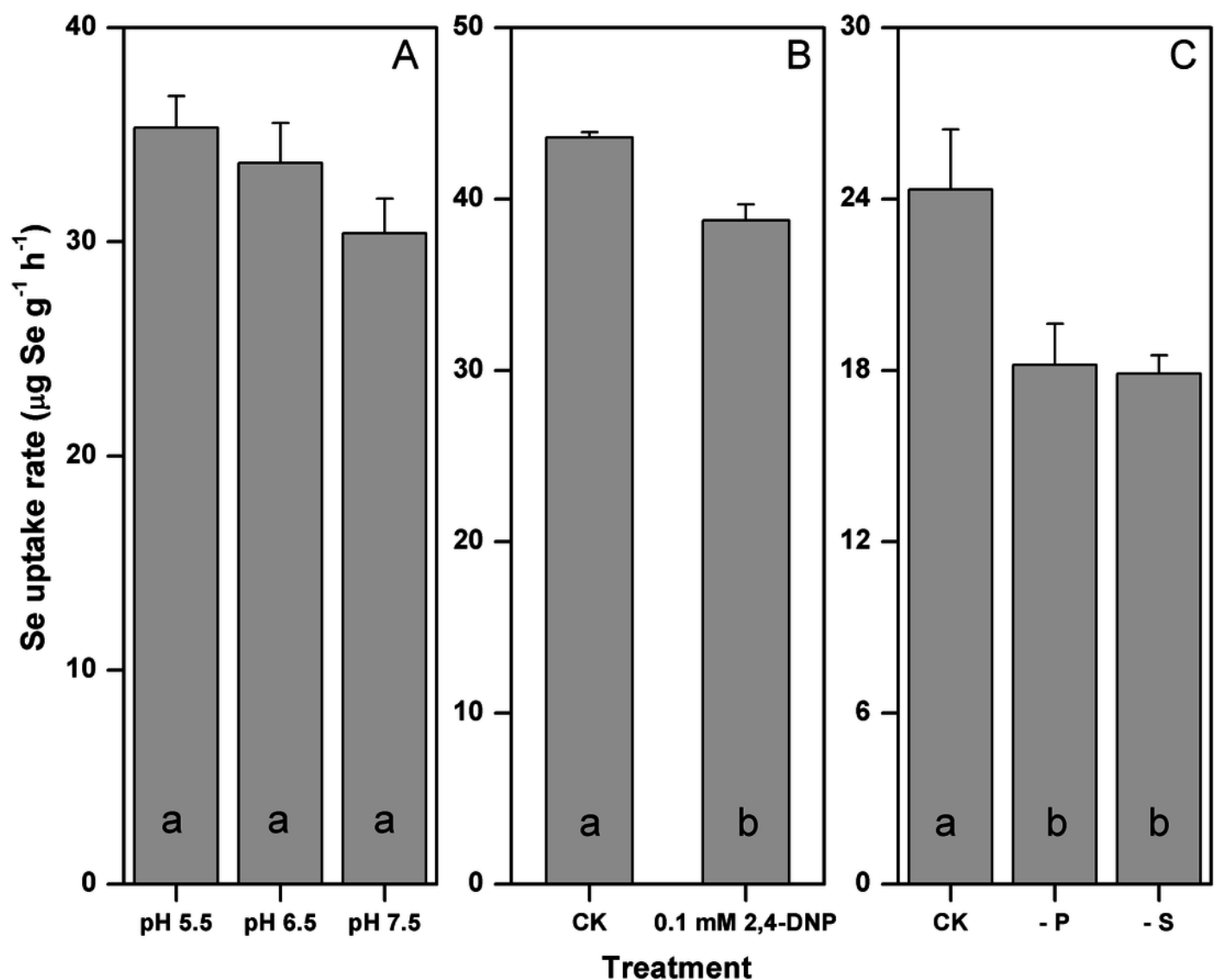
502   selenite uptake in rice. *Plant Physiology* 153:1871-1877.

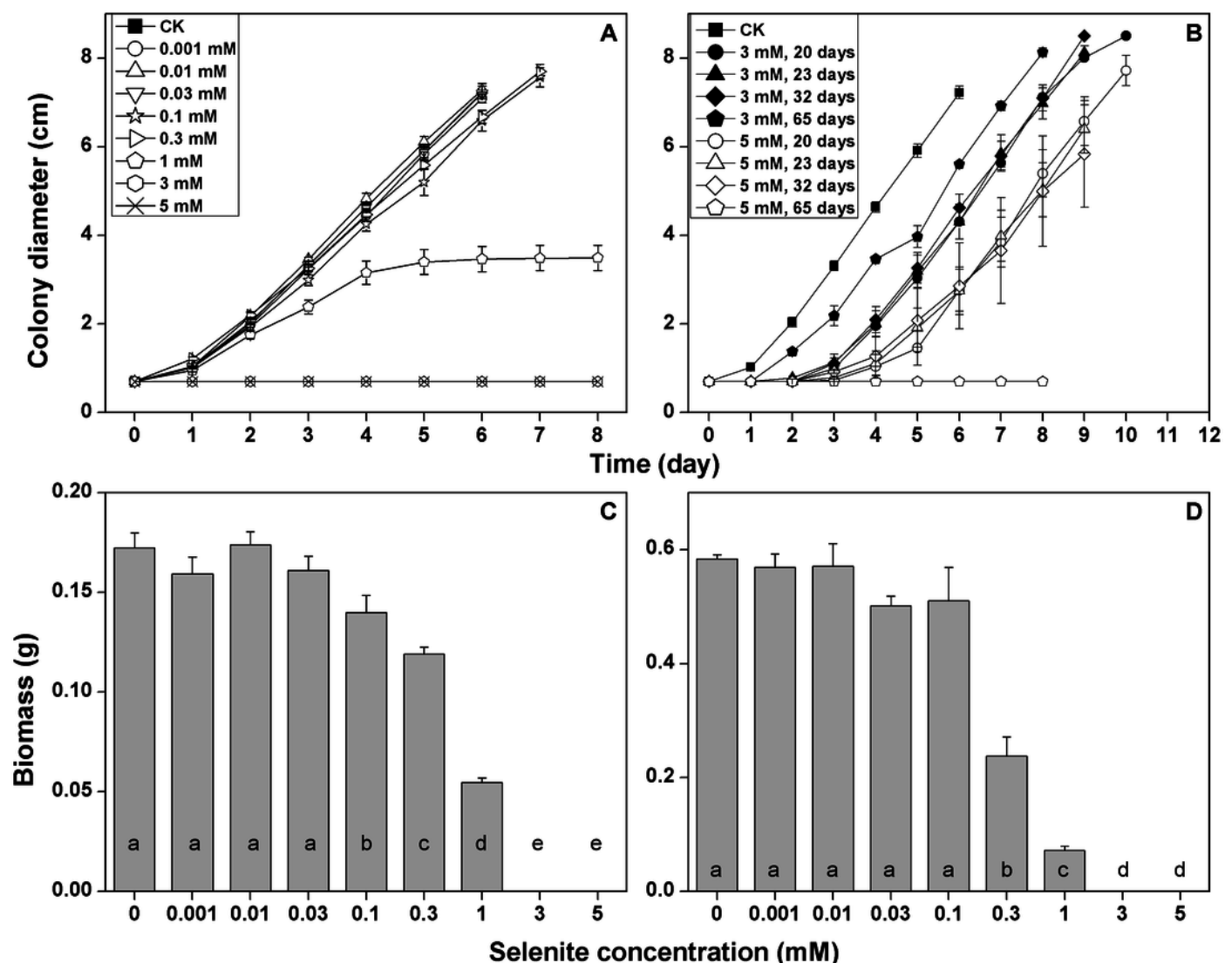
503   Zhu YG, Pilon-Smits EA, Zhao FJ, Williams PN, and Meharg AA. 2009. Selenium in higher plants: understanding

504   mechanisms for biofortification and phytoremediation. *Trends in Plant Science* 14:436-442.

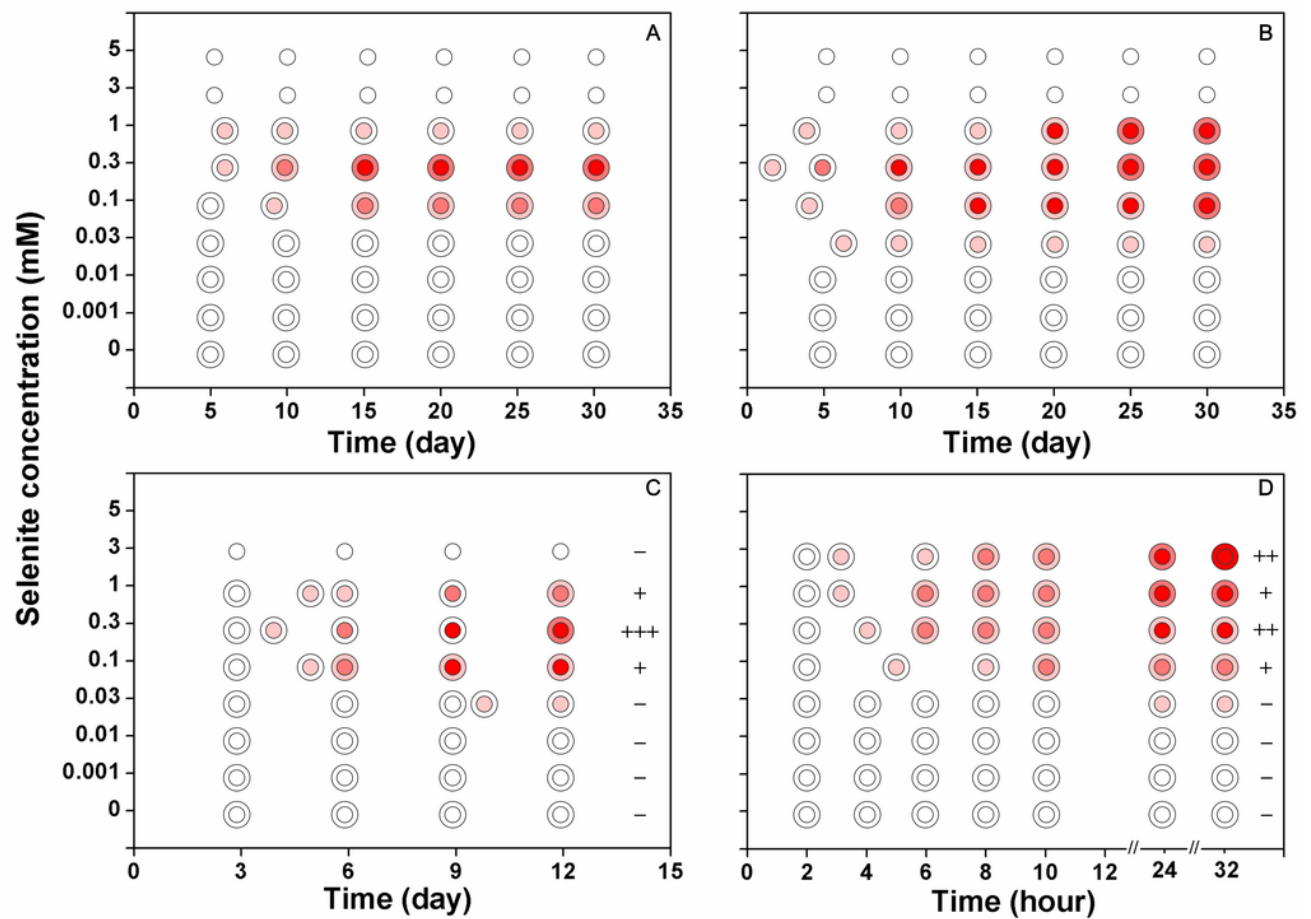
1

Influences of pH (A), metabolic inhibitor (B) and P or S starvation (C) on the selenite uptake by *F. velutipes.*

Different letters indicate statistical difference at the 0.05 level.

Growth responses of *F. velutipes* to 0-5 mM selenite in solid cultivation (A and B), static cultivation (C) and shaking cultivation (D).

In panel (D), the inocula were exposed to 3 mM or 5 mM selenite in solid cultivation for 20-65 days and then were inoculated onto the selenite-free media. Different letters indicate statistical difference at the 0.05 level.

3

The reduction intensities of *F. velutipes* supplied with 0-5 mM selenite (indicated by the red colorations of the colonies).

The isolates from the stock colonies were inoculated into the media containing 0-5 mM selenite and were subjected to solid cultivation (A), static cultivation (B) and shaking cultivation (C). In another experiment (D), after shaking cultivation in selenite-free media for 14 days, the resulting mycelial pellets were treated with 0-3 mM selenite. The first pink circle indicates the beginning of the red coloration. The small circle inside represents the central part of the colony (A and B) or the large mycelial pellet (C and D), and the ring outside represents the margin of the colony (A and B) or the small mycelial pellet (C and D). The plus/minus signs on the right side of circles in panels (C) and (D) represent the intensities of garlic smell after selenite treatment (−: none; +: low; ++: moderate; +++: strong).

TEM (A) and EDX (B) images of the hyphae of *F. velutipes* after selenite treatment.

The mycelia were picked up from the red region of the 20-day-old colony in the static cultivation supplied with 0.3 mM selenite.