Acta Physiol Plant (2012) 34:139–150
DOI 10.1007/s11738-011-0812-y

ORIGINAL PAPER

Paclobutrazol and plant-growth promoting bacterial endophyte *Pantoea* sp. enhance copper tolerance of guinea grass (*Panicum maximum*) in hydroponic culture

**Wei Huo · Chun-hua Zhuang · Ya Cao · Meng Pu ·
Hui Yao · Lai-qing Lou · Qing-sheng Cai**

Received: 18 August 2010 / Revised: 9 June 2011 / Accepted: 5 July 2011 / Published online: 20 July 2011

Abstract As most gramineous plants, guinea grass (*Panicum maximum*) comprise cellulosic biomass, which may be used as a feedstock for bioenergy. In order to develop such potential energy plants on copper-polluted lands, the hydroponic experiments with Cu, Paclobutrazol (PP333, a kind of antigibberellin) and plant growth-promoting bacterial endophyte (PGPB) treatments were carried out in a greenhouse. The seedlings of two cultivars of guinea grass, GG1 (*P. maximum var.* Natsukomaki) and GG2 (*P. maximum var.* Natsukaze) in 3 weeks old were treated, respectively, with different Cu treatments [0(CK), 100, 200, 300, 400, 500 μM l^{-1} Cu] for estimating Cu toxicity. The results showed that elevated Cu restrained plant growth and reduced biomass. According to the EC50 value [the Cu concentration when the relative gain in fresh weight ratio was 50% of control] of two tested cultivars, the concentration of Cu for further experiments was decided as 300 μM l^{-1}. Both pretreatments of PP333 (200, 400, 600 mg l^{-1}) and PGPB (*Pantoea sp.*) significantly alleviated the negative affect caused by stress of 300 μM l^{-1} Cu. The pretreatment of 400 mg l^{-1} PP333 promoted both two cultivars in biomass, compared to 300 μM l^{-1} Cu treat. The inoculation of *Pantoea* sp. Jp3-3 increased shoot dry weight, compared to Cu treat. The results suggested that the main reason for both PP333 and *Pantoea sp*. Jp3-3 enhanced Cu tolerance in guinea grass was that their pretreatments significantly decreased Cu absorption and accumulation under excessive Cu stress. The present study has provided a new insight into the exploitation of energy plant in heavy metal polluted condition by the way of plant growth regulation for increasing heavy metal tolerance.

Keywords Copper · Energy plant · Guinea grass (*Panicum maximum* var.) · Paclobutrazol (PP333) · *Pantoea* sp · Plant growth-promoting bacteria (PGPB)

Introduction

Metal mining and smelting, and the abuse of pesticides have caused severe heavy metal pollution to the environment (Bhuiyan et al. 2010), and then caused land degradation, which is a worldwide menace both for food security and human health (Nouairi et al. 2009). Among heavy metals, copper (Cu) is a required microelement for plant, but excess is highly toxic. A concentration of Cu in soils, that exceeding the maximum allowable concentration that required as nutrients or background levels, results in an accumulation of Cu in plants on deleterious levels (Srivastava et al. 2008). Excess copper could seriously inhibit the synthesis of chlorophyll and protein as well as carbohydrate and the uptake of phosphate and nitrogen (Xing et al. 2010). An important feature of copper toxicity is the formation of damaging oxygen radicals, and then causes increasing of MDA and proline contents, electrical conductivity and antioxidant enzyme activities (SOD, CAT, APX) (Li et al. 2009a).

By the crops from contaminated soils, excessive heavy metals enter the food chain and therefore do harm to human

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Communicated by B. Barna.

W. Huo · C. Zhuang · Y. Cao · M. Pu · H. Yao · L. Lou
College of Life Sciences, Nanjing Agricultural University,
Nanjing 210095, China

Q. Cai (✉)
Department of Plant Science, College of Life Sciences,
Nanjing Agricultural University, Nanjing 210095, China
e-mail: plant_sci@njau.edu.cn; qscai@njau.edu.cn

health (Lamb et al. 2009; Rahman et al. 2009). On the light of this, grain crops should be excluded from phytoremediation, although it is one of the most cost-effective and environment-friendly strategies of remediating metal-contaminated soils. Foregone hyperaccumulators, however, are known of their uneconomical usage, for almost all of them are at low biomass and slow growth velocity (Zhao et al. 2006). This shortcoming may also cause energy waste in disposal of the plant corpus. In recent years, developing energy plant in metal contaminated soils is considered as a corking solution to these problems (Shi et al. (2009). Energy plants can be used as producing biodiesel or bioethanol, which is a sustainable approach for the removal of metal pollutants by phytoremediation (Shi and Cai 2009). The tropical forage guinea grass (*Panicum maximum* var.), which has been widely used, is a common feed species (Ram 2009). Guinea grass behaves as high biomass, high growth rate and low humidity content, is a potential energy plant remain further study.

Paclobutrazol (PP333) is well-known as an antigibberellin plant growth regulator. Foliar applications of PP333 can reduce the top growth advantage apparently with few adverse effects on cropping (Blank et al. 2009; Li et al. 2009b). PP333 also increases the number of tillers, accelerates root growth, shortens internodes, promotes plant resistance to stress and improves crop yields (Mahoney et al. 1998; Sharma and Awasthi 2005; Thakur et al. 2006). Since invented in eighties of the last century, PP333 has been widely applied for both increasing in yield and promoting resistance (Chen et al. 2010).

Endophytic bacteria reside within plant hosts without causing disease symptoms (Taghavi et al. 2010). Endophytic bacteria have been found in virtually every plant studied, where they colonize the internal tissues of their host plant and form a range of different relationships (Rajkumar and Freitas 2008; Ryan et al. 2008). Among them endophytic plant growth-promoting bacteria (PGPB) could enhance host growth by phosphate solubilization activity (Rueda-Puente et al. 2010), osmotic adjustment, indole acetic acid production and the production of siderophore (Ma et al. 2009).

Previous studies have shown that guinea grass was able to ensure high yield. However, no studies have examined their growth and biomass performance as potential energy plant under copper (Cu) stress. Thus, the aim of this study was to (1) test Cu tolerance of the two cultivars of guinea grass, find a better candidate which is more tolerant to Cu, (2) application of PP333 to enhance Cu tolerance of the two cultivars, investigate the physiological mechanism of the enhancement, (3) elucidate the effects of PGPB on the plant growth and the uptake of Cu by the two cultivars. This study will establish a meaningful base for developing bioenergy feedstock resources cultivated in the areas polluted by excess heavy metal Cu by exploring bio-regulate way.

Materials and methods

Materials and seedling cultivation

The seeds of two guinea grass cultivars, GG1 (*P. maximum var*. Natsukomaki) and GG2 (*P. maximum var*. Natsukaze) (Snow Brand Seed Co., Ltd., Japan) were, respectively, surface-sterilized in 0.1% $HgCl_2$ for 15 min, rinsed with sterile water, soaked in 10% KNO_3 for 12 h. Then the seeds were sowed in culture dishes (Diameter 9 cm) filled with 5 g sterile clean river sand. At 10 days after germination in 12 h dark at 20°C, 12 h light at 27°C, and humidity about 50 ± 20%, uniform seedlings were selected and cultured in plastic cup (350 ml) filled with 400 g river sand, which was sterilized with 5% H_2O_2 for 30 min, and full rinsed by sterile water, and then 170°C parched for 4 h in oven before used. And every six cups were gathered in a plastic pot submersed with 1.0 L Hoagland solution (pH 5.8) and placed in growth-chamber (52 × 51 × 102 cm). At 3 weeks after cultivation in the cup, when the fifth leaf of the seedling fully expanded, uniform seedlings were transplanted into hydroponics plastic pots (22 × 16 × 7.5 cm). After 3 days buffer period, different treatments were conducted.

All the treatments as below were set in triplicate repetitions with three pots filled with 2.0 l Hoagland solution (pH 5.8), and 15 uniform seedlings were allowed to grow in each pot, at a uniform spacing. The pot trials were conducted in a greenhouse (30 ± 5°C, humidity of 50 ± 20%).

The plant growth under several treatments (Table 1)

Copper treatments

Six treatments of Cu [0(CK), 100, 200, 300, 400, 500 mol l^{-1}, supplied as $CuSO_4 \cdot 5H_2O$ were conducted for examining Cu tolerance of two grasses. The solution was refilled every 3 days. 12 days later, data were measured as described in "Measurements for plant growth, and physiological and chemical analysis".

PP333 application before Cu stress

When the fifth leaves were fully unfolded, 0 (control), 200, 400, 600 mg l^{-1} PP333, supplied as 15% wettable powder (purchased from Jiangsu Academy of Agricultural Sciences, Nanjing, China) were foliar sprayed, respectively. Each pot was treated with 50 ml PP333 solution while

Table 1 The abbreviation of treatments and plant materials in this experiment

Abbreviation	Detailed meaning
Name of plant material	
GG1	*Panicum maximum* var. Natsukomaki
GG2	*Panicum maximum* var. Natsukaze
Control and treatments	
Control (CK)	0 [not adding Cu, Paclobutrazol (PP333) and endophytic plant growth-promoting bacteria (PGPB)]
Cu	Cu: 300 μmol l^{-1}
P200	PP333: 200 mg l^{-1}
P400	PP333: 400 mg l^{-1}
P600	PP333: 600 mg l^{-1}
P200-Cu	Cu: 300 μmol l^{-1}, PP333: 200 mg l^{-1}
P400-Cu	Cu: 300 μmol l^{-1}, PP333: 400 mg l^{-1}
P600-Cu	Cu: 300 μmol l^{-1}, PP333: 600 mg l^{-1}
PGPB	PGPB pretreatment
PGPB-Cu	Cu: 300 μM l^{-1}, PGPB pretreatment

50 ml sterile water for the control. At 12 days after PP333 spraying, eight treatments, control (Cu: 0 μmol l^{-1}, PP333: 0 mg l^{-1}), Cu (Cu: 300 μmol l^{-1}, PP333: 0 mg l^{-1}), P200 (Cu: 0 μmol l^{-1}, PP333: 200 mg l^{-1}), P200-Cu (Cu: 300 μmol l^{-1}, PP333: 200 mg l^{-1}), P400 (Cu: 0 μmol l^{-1}, PP333: 400 mg l^{-1}), P400-Cu (Cu: 300 μmol l^{-1}, PP333: 400 mg l^{-1}), P600 (Cu: 300 μmol l^{-1}, PP333: 600 mg l^{-1}) and P600-Cu (Cu: 0 μmol l^{-1}, PP333: 600 mg l^{-1}) were conducted. The concentration of Cu (300 μmol l^{-1}) was decided basted on the experimental results of series Cu treatments ("Copper treatments"). 12 days later, different parts of plant were sampled for testing as describe in "Measurements for plant growth, and physiological and chemical analysis".

Endophytic PGPB pretreated before Cu stress

The Cu-resistance PGPB endophyte strain, *Pantoea* sp. Jp3-3 (accession number: EU781540) was screened and provided by Prof. Xia-fang Sheng, College of Life Sciences, Nanjing Agricultural University, China. The endophytic bacteria strain was separated from crabgrass (*Digitaria sanguinalis* (L.) Scop.), which grown on Cu contaminated soil in Tangshan Town, Jiangning District, Nanjing, China. Its 16S ribosomal RNA gene partial sequence is available on http://misuse.ncbi.nih.gov/. The bacteria was pre-cultured in liquid LB medium in 28°C for 24 h, centrifuged (7,000 rpm, 10 min), washed, and resuspended in phosphate buffer (pH 7), OD = 0.8 at 600 nm.

For PGPB endophytic pretreatment, germinated seeds were soaked in the bacterial suspension for 4 h while in sterile water was used as control. Then all the seeds were moved into plastic cups.

When the control group seedlings were 40 cm high (3 weeks after seeding), all the plants were transplanted in plastic pots. Four treatments, control (Cu: 0 μmol l^{-1}, no PGPB pretreatment), Cu (Cu: 300 μmol l^{-1}, no PGPB pretreatment), PGPB (Cu: 0 μmol l^{-1}, PGPB pretreatment), PGPB-Cu (Cu: 300 μmol l^{-1}, PGPB pretreatment). Twelve days later, data were measured as described in "Measurements for plant growth, and physiological and chemical analysis".

Measurements for plant growth, and physiological and chemical analysis

The hydroponic plants were sampled after Cu treatment for 12 days. Plant height and root length were measured before the samplings were separated into shoot and roots, and washed by distilled water, dried at 75°C in oven for 1 day and weighed before analysis of Cu concentrations. Also biomass per plant was measured by weighing using an electronic balance.

The chlorophyll content of the first full expanded leaf (youngest and count from top to bottom) in vivo was measured by chlorophyll meter (SPAD-502, KONICA MINOLTA, Tokyo, Japan) (Uddling et al. 2007).

Root activity was measured by triphenyltetrazolium chloride (TTC) deoxidization intensity (Lin et al. 2001). All the absorbencies were measured using a spectrophotometer (UV-2450, SHIMADZU, Tokyo, Japan).

The calorific values of the dry plant samples were measured using a SXHW-III microcomputer automatic calorimeter (Qitian, China) at 20°C, after porphyrization, compression and weighed.

Dry plant samples (0.2 g) were digested in concentrated HNO_3–$HClO_4$ (Guarantee reagent, 85: 15, v/v) using a microwave laboratory system (Milestone Ethos T, USA). After defining volumes to 10 ml using deionized water, concentrations of heavy metals were determined using an atomic absorption spectrophotometer (AAS novAA 400, Analytic-Jena, Germany).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Tukey's HSD using SPSS 17.0. Differences were considered to be significant at $p < 0.05$ or $p < 0.01$.

Results and analysis

Effect of Cu stress on the growth of two guniea grass cultivars

Growth parameters of GG1 and GG2 seedlings under Cu concentration gradient

The growth of GG1 and GG2 was suppressed, both their length and biomass of shoot and root decreased when the treated Cu concentration increased (Figs. 1, 2). Plants exposed to Cu in the concentration of 100 and 200 μM l^{-1} did not show significant inhibition in the shoot length compared to control, but root length was significantly inhibited under the Cu concentration of 100 μM l^{-1}. It was also shown that roots were more sensitive than shoots under heavy metal stress because of the immediate contact (Hernandez and Pastor 2008).

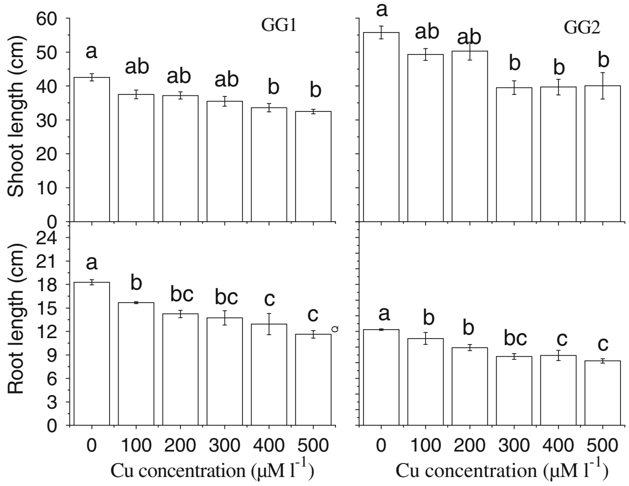

Fig. 1 Shoot and root length of GG1 (*Panicum maximum* var. Natsukomaki) and GG2 (*Panicum maximum* var. Natsukaze) when exposed to different concentrations of Cu for 12 days. The data are mean ± SE ($n = 9$). *Different letters* mean significant differences ($p < 0.05$) according to the Tukey HSD test

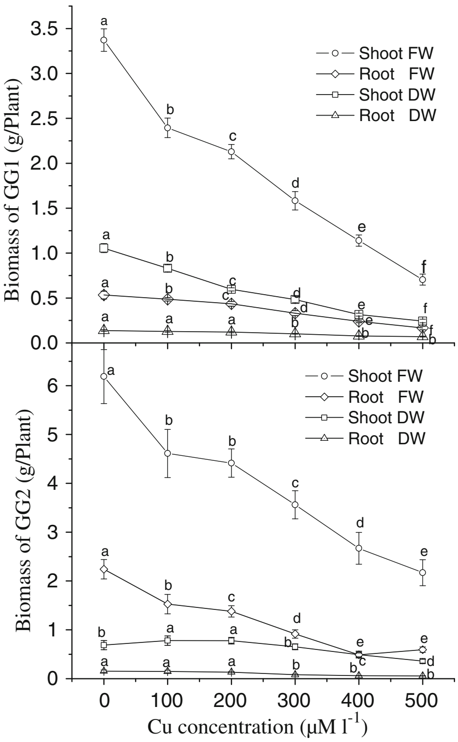

Fig. 2 Biomass of GG1 (*Panicum maximum* var. Natsukomaki) and GG2 (*Panicum maximum* var. Natsukaze) when exposed to different concentrations of Cu for 12 days. The data are mean ± SE ($n = 9$). *Different letters* mean significant differences ($p < 0.05$) according to the Tukey HSD test. *FW* fresh weight, *DW* dry weight

The relative gain in fresh weight ratio (RGW, similar to the tolerance index; Wilkins 1957) was used to characterize the degree of inhibition of plant growth by Cu stress, which was expressed as:

$$\mathrm{RGW} = T_{(W-W0)}/C_{(W-W0)} \times 100\% :$$

T treatment groups, *C* control groups, *W* final weight, *W0* initial weight.

The Cu concentrations (X) were related to fresh weight (Y) by regression analysis. The EC50, defined as the Cu concentration when the RGW was 50%, was used to characterize the tolerance to Cu stress (Table 2). According to the results shown, the EC50 values were 245.89 μM l^{-1} Cu for GG1 and 308.59 μM l^{-1} Cu for GG2, which suggest that GG2 was more tolerant to Cu than GG1.

Plants exposed to Cu treats in 100 and 200 μM l^{-1} did not show obvious inhibition in the root dry weight, compared to control. However, the root biomass was reduced and no significant difference was found among treats of 300, 400, 500 μM l^{-1} Cu. The stimulation in shoot dry weight of GG2 was found at 100 and 200 μM l^{-1} Cu concentrations. As for shoot, GG1 was significantly reduced in 100 μM l^{-1} Cu, and continuous decreased in higher Cu concentrations. As an essential element, lower

Table 2 Regression equation and EC_{50} values for the plants (based on fresh weight)

Plant species	Regression equation	R^2	EC_{50} (μmol/l)	95% confidence interval
GG1	$Y = 5.523E - 06X^2 - 0.01X + 4.353$	0.986	245.89	230.73−259.92
GG2	$Y = 10^{-5}X^2 - 0.0161X + 8.246$	0.972	308.59	293.16−324.07

The data are mean ± SE ($n = 9$)

EC_{50} values, the Cu concentration when the relative growth weight (RGW) was 50% of control

GG1 Panicum maximum var. Natsukomaki, *GG2 Panicum maximum* var. Natsukaze

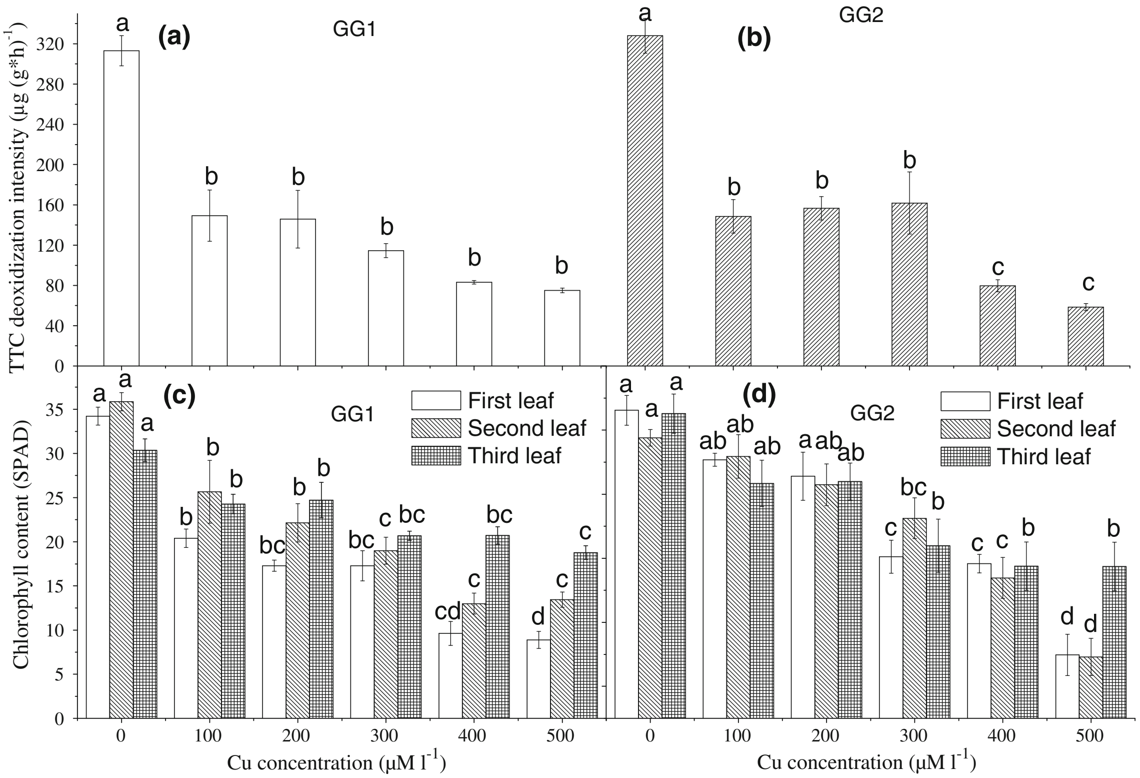

Fig. 3 Root activity and chlorophyll content of GG1 (*Panicum maximum* var. Natsukomaki) and GG2 (*Panicum maximum* var. Natsukaze) when exposed to different concentrations of Cu for 12 days. The first leaf was the youngest. The data are mean ± SE ($n = 9$). *Different letters* mean significant differences ($p < 0.05$) according to the Tukey HSD test

Cu concentration often stimulates plant growth, this is also observed in hypocotyls and radicals length (Lou et al. 2004). GG2, which was more tolerant according to fresh weight, performed better in shoot dry weight. Low Cu concentrations (100, 200 μM l^{-1}) even increased GG2 shoot dry weight by 13.41 and 12.87%, respectively, and high Cu concentrations decreased it gently. And then, 300 μM l^{-1} Cu was selected as a representative concentration for further treatments.

Physiological indexes of guinea grass under Cu stress

Root activity and chlorophyll content of GG1 and GG2 were shown in Fig. 3. These two physiological characters were positively related with the biomass. Root activity was significantly inhibited in 100 μM l^{-1} Cu, and showed a gradual trend to decrease in higher Cu concentrations ($p < 0.05$). Chlorophyll content also showed a continuous decreasing, and the youngest leaves (count from top to bottom) were more sensitive to Cu than the old leaves.

Alleviate effects of PP333 pretreatments on Cu toxicity to GG1 and GG2

Effect of PP333 on the growth of GG1 and GG2

It is showed that onefold treatment of PP333 and pretreatments of PP333 before Cu stress promoted growth of

GG1 and GG2, according to their growth parameters, length and biomass of root and shoot in Tables 3 and 4, respectively. Especially, after 12 days different Cu treatment, such promoting effects in pretreatments of PP333 before Cu stress were more significant ($p < 0.05$) in shoot length and shoot weight than in root length and root weight, especially in GG1. Anyway, pretreatments of PP333 had alleviated effects on Cu toxicity. Among the three pretreatments of PP333, under comprehensive consideration in contain economic efficiency; P200 was the most effective dosage.

In addition, there existed different effects of Cu and PP333 to growth of GG1 and GG2. In P200 and P200-Cu, Cu reduced GG1 shoot fresh weight by 10.3% while GG2 by 19.4%. In P400 and P400-Cu, these reduce rates were 14.8 and 35.8%, and 18.4 and 46.2% in P600 and P600-Cu.

Table 3 Effects of PP333 pretreatment on the length of shoot and root of guinea grass under Cu stress for 12 days

		Natsukomaki (GG1)		Natsukaze (GG2)	
		Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)
Control		76.4 ± 2.01abc	23.25 ± 1.56a	73.60 ± 2.77a	23.43 ± 0.73ab
Cu 300 μM l^{-1}		58.45 ± 3.90d	17.43 ± 2.16b	37.48 ± 2.55c	18.17 ± 1.20b
PP333 (mg/l)	Cu (μM l^{-1})				
200	0	88.45 ± 4.60a	24.03 ± 1.98a	84.28 ± 3.33a	24.38 ± 2.89ab
400	0	89.60 ± 2.77a	26.73 ± 1.22a	77.56 ± 4.39a	20.01 ± 1.14b
600	0	81.02 ± 4.11ab	26.13 ± 2.41a	75.12 ± 1.57a	27.65 ± 1.50a
200	300	80.4 ± 2.22ab	22.21 ± 1.82a	57.50 ± 2.72b	20.15 ± 0.74b
400	300	74.2 ± 2.17bc	24.63 ± 2.22a	51.21 ± 3.69b	24.68 ± 1.50ab
600	300	65.8 ± 3.63 cd	24.56 ± 0.53a	51.83 ± 3.3b	22.67 ± 1.75ab

The data are mean ± SE ($n = 9$). Different letters mean significant differences ($p < 0.05$) according to the Tukey HSD test

Table 4 Effects of PP333 pretreatment on the biomass of shoot and root of guinea grass under Cu stress for 12 days

		Shoot		Root	
		Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
GG1 (*Panicum maximum* var. Natsukomaki)					
Control		8.69 ± 1.39bc	1.22 ± 0.13bc	1.61 ± 0.29bc	0.22 ± 0.03bc
Cu 300 μM l^{-1}		5.01 ± 0.55c	0.69 ± 0.08c	0.99 ± 0.13c	0.12 ± 0.02c
PP333 (mg/l)	Cu (μM l^{-1})				
200	0	32.95 ± 7.51a	3.25 ± 0.8a	6.32 ± 1.67ab	0.58 ± 0.17a
400	0	36.42 ± 3.45a	2.95 ± 0.32ab	7.52 ± 0.78a	0.73 ± 0.05a
600	0	31.04 ± 5.32ab	3.51 ± 0.97a	6.05 ± 1.72ab	0.60 ± 0.13a
200	300	29.58 ± 4.18ab	2.97 ± 0.46ab	4.96 ± 0.85abc	0.52 ± 0.09ab
400	300	33.35 ± 5.4a	2.74 ± 0.49ab	5.53 ± 1.03abc	0.51 ± 0.11ab
600	300	25.38 ± 3.97abc	2.70 ± 0.48ab	5.36 ± 1.03abc	0.36 ± 0.07ab
GG2 (*Panicum maximum* var. Natsukaze)					
Control		13.35 ± 1.26bc	1.12 ± 0.12b	2.99 ± 0.36ab	0.33 ± 0.13a
Cu 300 μM		5.17 ± 0.49c	0.73 ± 0.07b	1.14 ± 0.18c	0.13 ± 0.02a
PP333 (mg/l)	Cu (μM)				
200	0	22.79 ± 3.14abc	2.39 ± 0.29ab	5.65 ± 0.65a	0.40 ± 0.06a
400	0	39.72 ± 9.20a	3.10 ± 0.70a	5.81 ± 1.19a	0.42 ± 0.10a
600	0	26.21 ± 3.47ab	2.18 ± 0.33ab	4.47 ± 0.65ab	0.28 ± 0.06a
200	300	18.37 ± 4.03bc	2.11 ± 0.48ab	2.59 ± 0.73bc	0.28 ± 0.07a
400	300	12.76 ± 3.83bc	2.15 ± 0.36b	2.97 ± 0.7ab	0.24 ± 0.08a
600	300	14.47 ± 2.86bc	2.36 ± 0.32b	2.99 ± 0.35ab	0.29 ± 0.06a

The data are mean ± SE ($n = 9$). Different letters mean significant differences ($p < 0.05$) according to the Tukey HSD test

These results strongly indicated that PP333 was more effective to GG1 than GG2, although GG2 biomass was still much higher than GG1 (Table 4).

Physiological effects of the PP333 pretreatment on copper stress

According to the results of regulating root activity by PP333 (Fig. 4a, b), all the pretreatments of PP333 before Cu stress (P200-Cu, P400-Cu and P600-Cu) showed their protective effects, but such effects were significant only in the treats at P200-Cu and P400-Cu for GG1. These results also strongly indicated that PP333 was more effective to GG1 than GG2. No significant effects of pretreatments of PP333 on chlorophyll contents in top three leaves were showed in comparing with Cu treat alone (Fig. 4c, d).

As shown in Table 5, under onefold treatment of PP333 (P400), the Cu contents in shoot and root were significant lower than control (except in shoot of GG2), while no such significant difference found in P200 and P600. Furthermore, under the pretreatments of PP333 before Cu stress (P200-Cu, P400-Cu and P600-Cu), the Cu contents both in shoot and root in two cultivars were significantly reduced ($p < 0.05$), compared to 300 μM l^{-1} Cu treat. And the Cu contents of roots in GG1 and GG2 decreased to 31.9 and 49.2%, respectively, in P400-Cu, which may be the most effective PP333 dosage. For the Cu contents of shoot in GG1, no significant differences among P200-Cu, P400-Cu and P600-Cu were found. The Cu content in shoot of GG2 also decreased compared to Cu treat, but the least content was observed in P200-Cu. Therefore, the pretreatments of PP333 may be a feasible choice for reducing the Cu content of the plant tissues.

It suggested that the main reason for PP333 enhanced Cu tolerance in guinea grass was that the pretreatments of PP333 significantly decreased Cu absorption and accumulation under excessive Cu stress. Although PP333's effects on the distribution and accumulation of Cu in different parts of the plant, and the mechanisms, are still unknown, it can be surely confirmed that PP333 is an effectual regulator for reducing Cu uptake by plants, and finally alleviating Cu toxicity to the plant. .

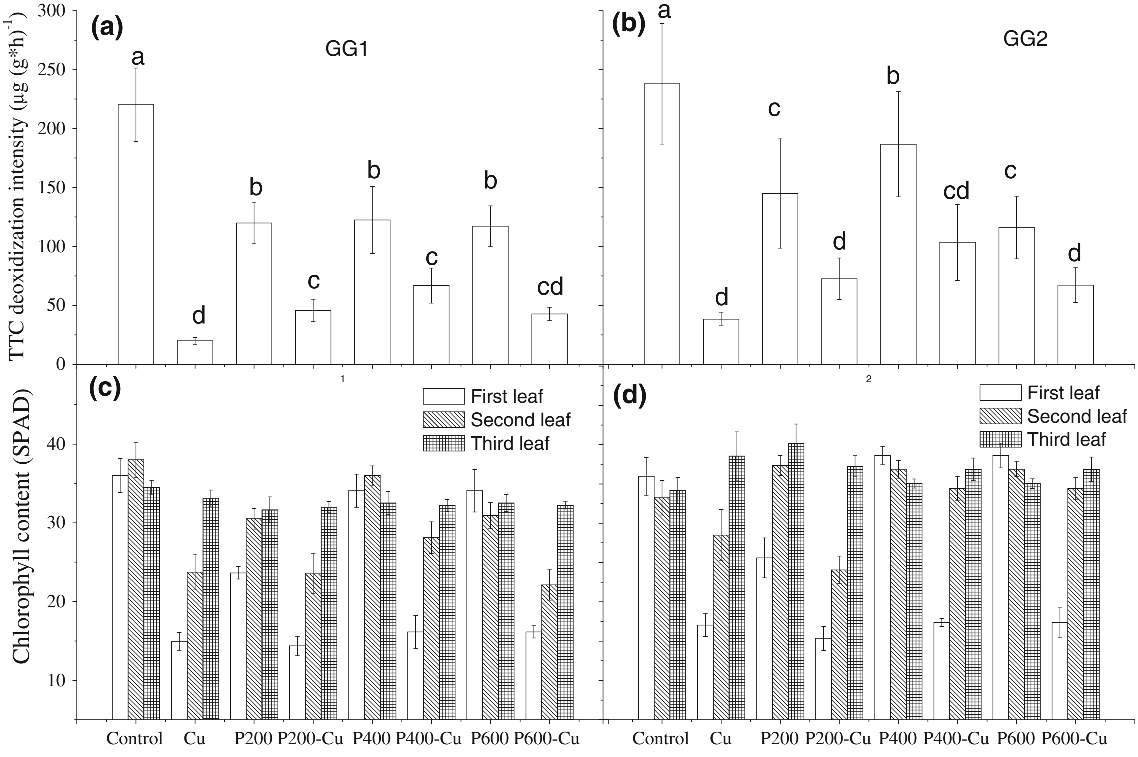

Fig. 4 Effects of PP333 pretreatment on root activity and chlorophyll content of guinea grass under Cu stress for 12 days. GG1 (*Panicum maximum* var. Natsukomaki) and GG2 (*Panicum maximum* var. Natsukaze). The data are mean ± SE ($n = 9$). *Different letters* mean significant differences ($p < 0.05$) according to the Tukey HSD test. Control (Cu 0 μM l^{-1}, PP333 0 mg l^{-1}), Cu (Cu 300 μM l^{-1}, PP333 0 mg l^{-1}), P200 (Cu 0 μM l^{-1}, PP333 200 mg l^{-1}), P200-Cu (Cu 300 μM l^{-1}, PP333 200 mg l^{-1}), P400 (Cu 0 μM l^{-1}, PP333 400 mg l^{-1}), P400-Cu (Cu 300 μM l^{-1}, PP333 400 mg l^{-1}), P600 (Cu 300 μM l^{-1}, PP333 600 mg l^{-1}), P600-Cu (Cu 0 μM l^{-1}, PP333 60 0 mg l^{-1})

Table 5 Effects of PP333 pretreatment on Cu content of guinea grass under Cu stress for 12 days

		Natsukomaki (GG1)		Natsukaze (GG2)	
		Shoot (mg/kg)	Root (mg/kg)	Shoot (mg/kg)	Root (mg/kg)
Control		17.96 ± 0.59c	84.90 ± 3.38d	12.39 ± 1.53f	94.23 ± 2.91f
Cu 300 μM l^{-1}		66.99 ± 0.71a	1404.75 ± 47.45a	60.80 ± 3.35a	1,370.1 ± 168.19a
PP333 (mg/l)	Cu (μM l^{-1})				
200	0	16.92 ± 0.78c	157.72 ± 24.65d	17.48 ± 0.77e	119.17 ± 10.92e
400	0	10.75 ± 0.88d	39.34 ± 9.63e	14.49 ± 1.83ef	70.12 ± 8.68 g
600	0	17.55 ± 2.23c	96.84 ± 19.56d	11.32 ± 0.61f	115.06 ± 13.68ef
200	300	31.52 ± 1.53b	553.38 ± 34.64b	20.73 ± 0.33d	765.95 ± 55.31c
400	300	30.99 ± 0.75b	436.68 ± 89d	27.32 ± 2.74c	600.83 ± 46.45d
600	300	31.88 ± 1.27b	472.75 ± 19.71c	33.97 ± 7.01b	899.08 ± 130.87b

The data are mean ± SE ($n = 9$). Different letters mean significant differences ($p < 0.05$) according to the Tukey HSD test

Influence of *Pantoea* sp. Jp3-3 on GG1 and GG2 growth under Cu stress

After inoculating *Pantoea* sp. Jp3-3 (PGPB), the fresh weights of shoot and root increased significantly (Fig. 5). Jp3-3 enhanced the fresh weights of shoot and roots by 217 and 202.2% in GG1, and by 240.1 and 228.8% in GG2, respectively, compared to the control. Under the pretreatment of inoculating *Pantoea* sp. Jp3-3 before Cu treat (PGPB-Cu), both the fresh weights and dry weights of shoot and root in GG1 and GG2 increased significantly, not only comparing with Cu treat, but also with control. These results indicated that Jp3-3 was an effective PGPB for GG1 and GG2, not only at normal condition, but also under Cu stress. In addition, such PGPB's effects alleviating Cu toxicity was more impactful to GG2 than GG1 (Fig. 5).

Under the pretreatment of inoculating *Pantoea* sp. Jp3-3 before Cu treat (PGPB-Cu), the Cu contents both in the root and shoot decreased significantly both in GG1 and GG2 (Fig. 6), compared to Cu treat. Cu content in PGPB-Cu group was 36.2% of the Cu treat in GG1 roots, and 36.8% in GG2 roots. As one kind of PGPB, Jp3-3 exhibited an obvious protection from absorbing excessive Cu by root firstly and transferring into shoots secondly. It also suggested that the main reason for PGPB enhanced Cu tolerance in guinea grass was that the pretreatments of PGPB significantly decreased Cu absorption and accumulation under excessive Cu stress.

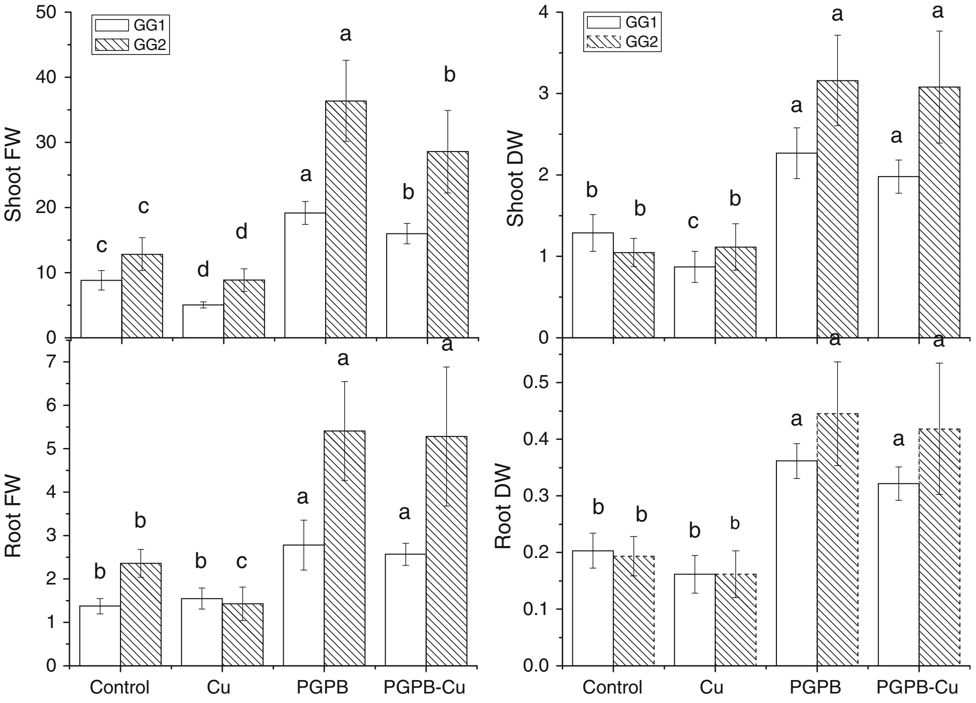

Fig. 5 Effects of PGPB pretreatment on biomass of guinea grass under Cu stress for 12 days. The data are mean ± SE ($n = 9$). *Different letters* mean significant differences ($p < 0.05$) according to the Tukey HSD test. Control (Cu 0 μM l^{-1}, no PGPB pretreatment), Cu (Cu 300 μM l^{-1}, no PGPB pretreatment), PGPB (Cu 0 μM l^{-1}, PGPB pretreatment), PGPB-Cu (Cu 300 μM l^{-1}, PGPB pretreatment). *FW* fresh weight, *DW* dry weight. GG1 (*Panicum maximum* var. Natsukomaki) and GG2 (*Panicum maximum* var. Natsukaze)

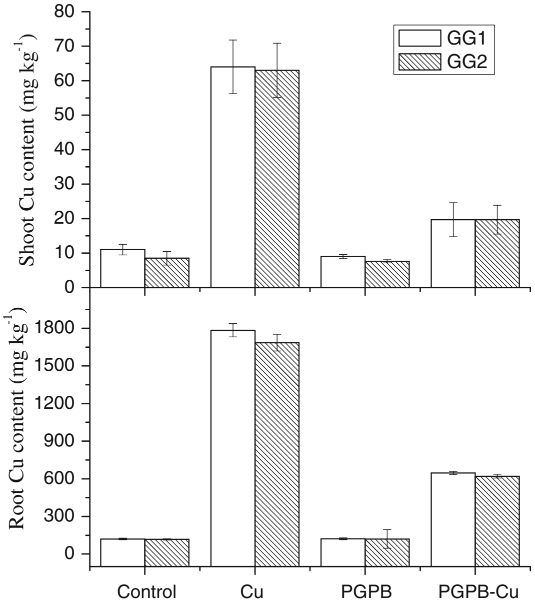

Fig. 6 Effects of PGPB pretreatment on Cu content of guinea grass under Cu stress for 12 days. The data are mean ± SE ($n = 9$). Control (Cu 0 μM l^{-1}, no PGPB pretreatment), Cu (Cu 300 μM l^{-1}, no PGPB pretreatment), PGPB (Cu 0 μM l^{-1}, PGPB pretreatment), PGPB-Cu (Cu 300 μM l^{-1}, PGPB pretreatment). GG1 (*Panicum maximum* var. Natsukomaki) and GG2 (*Panicum maximum* var. Natsukaze)

Discussion

Energy plant is considered of multitudinous purposes such as being transformed into energy for heat and electric power production through direct combustion, used as a feedstock for liquid transportation fuel, or gasified and liquefied to produce syn-gas or bio-oil. Several perennial herbaceous grasses have been studied in recent years as potential dedicated cellulosic biomass crops (Bals et al. 2010; Keshwani and Cheng 2009). Switchgrass (*P. virgatum*) has been legalized as one of the most important bioenergy plants over the world, especially in USA (Parrish and Fike 2005). Guinea grass has similar characteristics to switchgrass, such as tolerant to abiotic stress, high biomass, low humidity content, abundant cellulose and low requirements for agricultural inputs (Ram 2009; Carvalho et al. 2006). Previous studies were focused on its high productivity, but an important prerequisite for high productivity is the suitable soil conditions (Aylott et al. 2008). However, the use of arable land for bioenergy production has been criticized as diverting material away from the human food chain, with resulting of food shortages and price rise (Escamilla-Trevino et al. 2010). Meanwhile, the continued industrialization of countries has led to extensive environmental problems. A wide variety of chemicals (e.g. heavy metals, pesticides, chlorinated solvents, etc.) have been detected in soil and water (Rajkumar and Freitas 2008; Zhao et al. 2007). Among the pollutants, heavy metal is an important reason contributing to land deterioration, especially in mine-affected agricultural soils.

Generally, elevated levels of Cu are known to exhibit harmful effects on plant growth. At high concentrations, Cu inhibits seed germination, reduces plant growth, decreases enzyme activity of metabolism in plants and even causes plant death (Brewin et al. 2003). The present results showed that both GG1 and GG2 were tolerant to low concentration of Cu, especially GG2. Cu at the level of 200 μM l^{-1} did not significantly inhibit the shoot length of GG1 and GG2 in comparison with the control. However, as the Cu concentrations in the solution increased continually, plant growth was inhibited, according to the results of length and biomass of shoot and root in GG1 and GG2. Therefore, it is necessary to find a way to increase the Cu tolerance of guinea grass.

Paclobutrazol (PP333) is generally applied as root and foliar treatments (Wang et al. 1986). Previous researches were focused on its production increasing and water and salt stress alleviation effects (Dubey et al. 2009; Lin et al. 2006). It was found that PP333 provided an average reduction of banana in pseudostem height of 26%, thus promoted the yield of bananas (Maia et al. 2009). In another research, PP333 significantly increased the root and stem length, total leaf area, fresh weight, dry weight and activities of antioxidant enzymes, thus ameliorated the adverse effects of NaCl stress in *V. unguiculata* plants (Manivannan et al. 2008). Under water stress, PP333 exhibited a significant promotion of groundnut (*Arachis hypogaea* L.) growth by increasing the antioxidant levels and activities of scavenging enzymes such as SOD, APX and CAT (Sankar et al. 2007). Besides, other studies also proved that PP333 could increase plant growth under stress (Hajihashemi and Kiarostami 2007; Jaleel et al. 2007). Our studies provided a new regulate function of PP333 here. PP333 significantly increased biomass and root activities of two cultivars of guinea grass (GG1 and GG2) even under stress of 300 μM l^{-1} Cu, compared with the control. Although the cellula interaction mechanism of PP333 and Cu was still unknown, it was clear that PP333 exhibited a definite positive effect to the plant under heavy metal stress. Besides, there was littlereports on the effect of PP333 to plant heavy metal contents. PP333 reduced the potassium and magnesium contents of potato tubers (Tekalign and Hammes 2005), decreased Cu content in peach trees (Blanco et al. 2002). In our study, all the pretreatments of PP333 significantly reduced Cu contents of shoots and roots in 300 μM l^{-1} Cu. These results indicated that PP333 could decrease root uptake of Cu, thus decrease the shoot Cu content, and finally caused to alleviate stress of high concentration of Cu.

The endophytic PGPB promoted the growth of two cultivars of guinea grass (GG1 and GG2) not only under Cu

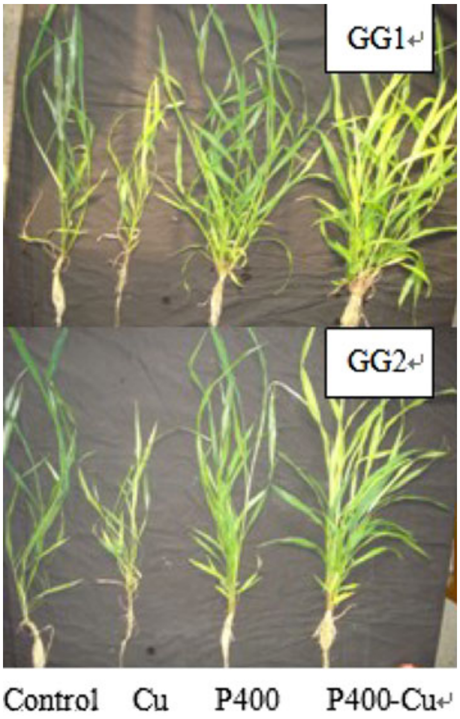

Fig. 7 PP333 increased tiller number of GG1 (*Panicum maximum* var. Natsukomaki) and GG2 (*Panicum maximum* var. Natsukaze)

stress, but also in non-Cu treat. There were no significant differences in dry biomass between PGPB and PGPB-Cu (Fig. 5). However, as for PP33, which markedly increased tiller number of both cultivars (Fig. 7, data not shown), this might be a major reason for PP33 pretreatment to biomass increasing under Cu stress. As for endophytic PGPB, which promote plant growth caused by a number of similar mechanisms. These include phosphate solubilization activity indole acetic acid production and the production of a siderophore (Verma et al. 2001). *Pantoea* sp. is an endophytic nitrogen-fixing bacterium isolated from sugarcane tissues (Loiret et al. 2004). *Pantoea* sp. showed nitrogenase activity in 5 mmol L^{-1} of serine, asparagine, threonine, alanine, proline, tyrosine, valine, methionine, lysine, phenylalanine, cysteine, tryptophan, citrulline and ornithine (Loiret et al. 2009). The increase in amino acid contents as result of *Pantoea* sp. inoculation could be related to biological N fixation, metabolic response of the plant or bioactive molecules secreted from the bacteria. *Pantoea* sp. 18-2 was also found to effectively reduce the acetylene in cultivated rice (*Oryza sativa* cv. Nipponbare) and wild rice (*O. officinalis*) (Zakria et al. 2008). Another research reported that *Pantoea agglomerans* could help in biocontrol of banana pathogens. Our results firstly showed PGPB activities of *Pantoea* sp. in heavy metal resistance. Besides, certain endophytic bacteria can surely improve host plant's resistance to heavy metals and application of endophytic bacteria was found to enhance uptake of minerals and concentration of heavy metals (Rajkumar et al. 2009). However, in our present study, *Pantoea* sp. Jp3-3 significantly reduced Cu uptake by guinea grass, and the efficiency of reduction was similar to PP333 (Fig. 7). Since excessive metals in plant cells inhibited enzyme activities, thus put adverse effects on metabolism (Tandy et al. 2009), therefore, we can concluded that the main regulating function both for PP333 and PGPB alleviated the stress caused by excessive Cu to the plants of guinea grass may be owing to inhibition of Cu uptake caused by pretreatment PP333 or PGPB.

Acknowledgments We thank the financial support from Natural Science Foundation of China (40971296) and Nanjing Agricultural University Student Research Training (SRT) Fund (0906A23). The authors thank Dr. Mike Zhang for his sincere advice during preparation and correction of the manuscript.

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