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RESEARCH PAPER

Effects of lead contamination on the clonal propagative ability of *Phragmites australis* (common reed) grown in wet and dry environments

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ABSTRACT

Clonal propagation is important for the survival and maintenance of the common reed *Phragmites australis*. Pot culture experiments were conducted to investigate the effects of lead (Pb) concentration (0, 500, 1500, 3000, 4500 mg·kg^{-1}) and water stress on the clonal reproductive ability of this species. The Pb concentration found in plant organs, in decreasing order, was roots >shoots >rhizomes. There was a negative relationship between the growth of clonal propagative modules (excluding axillary shoot buds) and Pb concentrations, which caused a decrease in biomass, rhizome growth and number of axillary and apical rhizome buds. Daughter axillary shoots exhibited a tolerance strategy, with no significant change in their number; the axillary and apical rhizome buds, daughter apical rhizome shoots and rhizomes exhibited compensatory growth during the late stage of Pb (excluding 4500 mg·kg^{-1}) treatment in a wet environment. Pb applications above 500 mg·kg^{-1} reduced these parameters significantly in the drought treatment, except for the number of axillary shoot buds, which did not change. Our results indicate that clonal propagative resistance to Pb contamination can occur *via* tolerance strategies, compensatory growth and a Pb allocation strategy, enabling these reeds to maintain population stability in wet environments. However, clonal modular growth and reproductive ability were inhibited significantly by the interaction between drought and Pb, which would cause a decline in *P. australis* populations in a dry environment. Lead concentrations of 4500 and 500 mg·kg^{-1} in soils might meet or exceed the Pb tolerance threshold of clonally propagated reeds in wet and dry environments, respectively.

INTRODUCTION

Heavy metal contamination is a serious problem in many areas around the world, and is difficult to remedy (Mohammed *et al.* 2011; Jiang *et al.* 2014). The levels of heavy metal contamination in soils range from trace to as high as 10,000 mg·kg^{-1} (Blaylock & Huang 2000). Lead (Pb) is a toxic heavy metal that has attracted more attention because of its long persistence in soil and high phytotoxicity. An appreciable quantity of Pb can be absorbed by plants through the roots system (Karataglis & Alexiadis 1982), and may restrict growth of plant organs, *i.e.* roots, shoots and leaves (Gopal & Rizvi 2008; Islam *et al.* 2008; Feigl *et al.* 2013), even resulting in stunted overall growth. In addition, lead can inhibit photosynthesis (Islam *et al.* 2008; Hattab *et al.* 2009; Mateos-Naranjon *et al.* 2011), disrupt antioxidant enzyme activity and increase lipid peroxidation caused by reactive oxygen species (ROS; Reddy *et al.* 2005; Hu *et al.* 2012; Wang *et al.* 2012). Such changes in key physiological processes eventually lead to a decrease in biomass (Reddy *et al.* 2005; Gopal & Rizvi 2008; Hu *et al.* 2012; Wang *et al.* 2012). However, some plants are able to tolerate or resist heavy metal exposure through specialised physiological mechanisms (Ruley *et al.* 2004; Hu *et al.* 2012) or external morphological changes (Baker 1987; McCue & Hanson 1990).

Clonal propagation enables plants to produce genetically identical daughter shoots with the potential to become independent of the mother organism (Klimeš *et al.* 1997). For plants that have predominantly clonal growth, reproduction from seed makes a negligible contribution to the number of offspring (Grace 1993; Eckert 2002), while clonal propagation is key for their maintenance. The clonal reproductive ability of plants after a disturbance is a complicated but important trait. There are two methods to evaluate this: (i) assess resprouting of buds after disturbance (Cornelissen *et al.* 2003); or (ii) count bud number as an indication of potential clonal reproduction (Dalgleish & Hartnett 2006). The belowground bud bank was first characterised by Harper (1977), and is defined as the population of meristems beneath the soil that are associated with rhizomes or other perennial organs. Recently, this has been expanded to

include all buds that can be potentially used for vegetative regeneration (Klimešová & Klimeš 2007). The crucial role of the bud bank after disturbance has been documented in numerous environments, such as those affected by fire, grazing, drought and flooding. Fernandes *et al.* (2008) noted that some plants with a large number of buds are more resistant to fire. Similarly, a large reserve population of belowground buds may improve resistance to and rate of recovery from drought, heavy grazing or other environmental stresses (Hartnett *et al.* 2006). For clonal plants, new shoots that resprout from the bud bank play a considerable role in the expansion of plants and the maintenance of population dynamics. The resprouting ability of buds may increase a plant's resistance to recurrent flooding disturbance (Mony *et al.* 2011), which facilitates stability of populations in aquatic ecosystems. Vegetative propagation of rhizomatous clonal plants mainly originates from rhizomes, which are progenitive fragments or structures. The survival and vegetative propagation from rhizomes may be hindered when plants are exposed to long-term heavy metal contamination (Fürtig *et al.* 1999), which can contribute to species decline. For rhizomatous clonal plants, clonal reproductive modules are composed of buds, daughter shoots and rhizomes, and changes in their abundance can greatly alter a plant's clonal propagative ability. The response of clonal reproductive modules may vary with the type of disturbance such that specific disturbances may encourage specific clonal reproduction traits. However, little research to date has focused on heavy metal impacts on the clonal reproductive ability and modules of clonal plants.

Phragmites australis is a typical rhizomatous perennial plant, whose extensive rhizomes (bearing buds) constitute a dense, intercommunication network from which plants emerge (Haslam 1969) to maintain population stability. Generally, rhizomes with very active metabolism, such as high antioxidant enzyme activity, are more resistant to harsh wetland environments and pollutants, including heavy metal contamination (Massacci *et al.* 2001; Fediuc & Erdei 2002). There is evidence that *P. australis* has extensive heavy metal tolerance (Ye *et al.* 1997; Ye *et al.* 1998) and the ability to remove heavy metals from soils, and can be used in phytoremediation (Fediuc & Erdei 2002; Iannelli 2002). This species has therefore been widely used in the construction and restoration of wetlands, and to treat industrial wastewater containing heavy metals and other pollutants. *P. australis* relies mainly on clonal propagation to colonise new space and maintain population stability in various environments. However, the growth response of its clonal propagules to Pb exposure and potential changes in clonal propagative ability have not been reported.

Wetlands are often characterised by relatively large seasonal fluctuations in water levels, being flooded and suffering high water saturation during some parts of the year while experiencing prolonged dry periods in others (Mitsch & Gosselink 2000). Little is known of how the clonal propagative ability and modules of reeds in wetlands might change with seasonal water fluctuations and Pb pollution, especially in terms of a comparison between moist and drought conditions. For soils with pH > 6.5, the toxicity threshold of soil Pb is 500 $mg \cdot kg^{-1}$ according to the environmental quality standard for soil, GB15618-1995, issued by the State Environmental Protection Administration of China. Typically, the tolerance of each plant species' to Pb can best be assessed using toxicity assays across a range of concentrations (Windham *et al.* 2001). Thus, we designed pot experiments to imitate moist and drought environments along a gradient of five Pb concentrations (0, 500, 1500, 3000 and 4500 $mg \cdot kg^{-1}$) to study the effects of Pb and water stress on clonal reproductive ability, and to assess tolerance limit to Pb in clonal reproduction of *P. australis*. Success was measured as the number of buds and daughter shoots, using various rhizome parameters (number of rhizomes, total rhizome length, number of rhizome nodes and rhizome internode length). The objectives of our study were to determine *P. australis* tolerance to Pb contamination under drought and well-watered conditions: (i) adaptive strategies of clonal reproductive modules; (ii) response of clonal propagative ability; (iii) any synergetic effects between Pb and drought; (iv) whether there is a tolerance threshold to Pb of clonal reproduction and, if so, and how well do *P. australis* grow in wet and dry environments.

Clonal propagation plays a vital role in the survival and succession of *P. australis* populations. Not only does our experiment represent the first study of the effects of Pb on the clonal reproductive ability and modules of *P. australis*, but also furthers study of the interactive effects of Pb and water stress. We provide new insights into the influence of heavy metals on clonal propagation of *P. australis* in wet *versus* dry environments, and the fundamental role of clonal propagation in the spread and maintenance of *P. australis* populations growing in different habitats under heavy metal stress.

MATERIAL AND METHODS

Preparation of experimental material

Seeds of *P. australis* were collected from mature wild plants in a wetland that had ample water levels during the summer but were relatively dry in other seasons. The wetland is located west of Changchun city, Jilin province, China (125°1′E, 43°56′N, 188 m a.s.l.). Soil used in the pot experiment was collected from the surface layer (0–20 cm) of a grassland near the Northeast Normal University field experiment station, in Changling County, Jilin Province, China (123°44′E, 44°44′N, 167 m a.s.l.). Soil total nitrogen (N) was 6.9%, organic carbon 0.4%, pH 8.6, electrical conductivity 91 $\mu s \cdot cm^{-1}$ and field capacity of 200 $g \cdot kg^{-1}$. The fresh soil was mixed homogeneously and allowed to air dry, then passed through a 1-mm sieve, after which it was divided into 10-kg subsamples. Specified concentrations of $Pb(NO_3)_2$ solution (1.4 l) were added and thoroughly mixed into the soil subsample to obtain five levels of Pb contamination: 0, 500, 1500, 3000 and 4500 mg $Pb \cdot kg^{-1}$. The relative water content of the spiked soils was 70% of field capacity. The contaminated soil subsamples were transferred into plastic pots (30-cm diameter × 35-cm height). Prior to addition of contaminated soil, the bottoms of 60 plastic pots used in the well-watered treatment were sealed with transparent tape to ensure that water and contaminated soil could not leak out. The remaining 60 pots for the drought treatment were lined with several layers of gauze to prevent contaminated soil from leaking out. Finally, all the soil-filled pots were placed in a darkened room with ventilation for 45 days (from 1 May to 15 June 2013). During this time, the spiked soils were not further mixed in order to obtain equilibration of amended soils

before the start of the experiment, following Jia *et al.* (2010). Small samples of spiked soil were analysed for Pb content and values were: 12.9 ± 0.32, 562 ± 8.97, 1574 ± 40.47, 3812 ± 140.91, 4750 ± 170.24 mg Pb·kg^{-1}, which was slightly higher than the five target levels of added Pb.

Plant cultures and treatments

Seeds of *P. australis* were sown in plastic tanks ($80 \times 50 \times 30$ cm) that contained 20 cm of humus soil, and watered daily to keep soil moist. The tanks were housed in a greenhouse at 12–24 °C, on the campus of Northeast Normal University, Jilin City, China (43°51′N, 125°19′E, 236 m a.s.l.). After 50 days had elapsed since sowing (from 25 April to 15 June 2013), uniform plants ~8-cm high, with four or five leaves, were transplanted into the pots, then placed in the greenhouse. As some weak seedlings were not expected to survive after transplantation, we initially place 20 seedlings into each pot, and allowed them to grow for 5 days under weak sunlight under shading with aster cloth. The weakest plants were then removed to retain ten strong seedlings in each pot. The experiment was arranged as a split-plot design with the two levels of water treatment as main factor, and five levels of Pb treatment for a total of ten treatments and 12 replicate pots per treatment. Pots were exposed to one of two water treatments: 'well-watered', with a 3–5 cm layer of water above the contaminated soil surface, and 'drought', with water levels at 50–55% of field capacity. Pots in the well-watered treatment were watered directly, and those in the drought treatment were watered to 11–11.1 kg, using an electronic balance (ACS-30; KaiFeng Group, Zhejiang, China), at 17:00 h each day. Temperature, light and humidity were not regulated (Ye *et al.* 1997, Ye *et al.* 1998), and maximum/minimum temperatures were 32/16 °C (day/night) during the treatment period of 90 days from 20 June to 20 September 2013.

Classification of belowground buds and aboveground daughter shoots

Plants that developed from seed were defined as 'parent shoots', and those that resprouted from buds of rhizomes or basal nodes of parent shoots were considered as 'daughter shoots'. The classification system for belowground buds and aboveground daughter shoots of a clonal plant, proposed by Zhang *et al.* (2009), was employed in the present work. The three categories of belowground buds were (i) axillary shoot buds, (ii) axillary rhizome buds and (iii) apical rhizome buds. The three categories of daughter shoots were (i) daughter axillary shoots, (ii) daughter axillary rhizome shoots and (iii) daughter apical rhizome shoots. The axillary shoot bud at the basal node of a shoot can grow upwards to form a daughter axillary shoot, or horizontally into a primary rhizome. The axillary rhizome buds attached to rhizomes grow upwards to form daughter axillary rhizome shoots, or horizontally into rhizomes, which we defined as secondary rhizomes. The apical rhizome buds originating from the end of a rhizome continue to form daughter apical rhizome shoots or grow horizontally to extend the rhizome.

Determination of clonal reproductive modules

Plants from four of the 12 replicate pots per treatment were randomly destructively sampled 30, 60 and 90 days after the start of treatment. Each plant was separated from the contaminated soil together with its root system. Then the number of each type of bud >1 mm in length, number of each type of daughter shoot, number of rhizomes, number of rhizome internodes and total length of all rhizomes per parent shoot was assessed. The rhizome internode length was calculated by dividing total rhizome length by number of rhizome nodes. The number of buds, daughter shoots and rhizome parameters (number of rhizomes, total rhizome length, number of rhizome nodes, rhizome internode length) were expressed per parent shoot. Finally, each plant was washed gently with tapwater, three times with deionised water and then divided into shoot, rhizome and root components before being oven-dried at 75 °C to a constant weight, and dry weight measured. The dried tissues were ground into fine powder with ball mill (MM400; Retsch, Haan, Germany) for measurement of Pb concentrations.

Measurement of Pb concentrations

The homogeneous soil samples and plant power (0.15 g) were digested in a microwave oven (CEM Mars5; Analyx, Wellesley, MA, USA) with solutions of 5:1 HNO_3:HF and HNO_3:$HCLO_4$ (v/v) for 120 min. The clear liquid was further digested in a heating digestion apparatus (DigiBlock ED36; LabTech, Cary, NC, USA) until approximate 0.5 cm in height at 180 °C. The cooled solution was made up to 50 ml total volume. Concentrations of Pb were determined using a graphite furnace atomic absorption spectrometer (SpectrAA Z220; Varian, Palo Alto, CA, USA). The detection limits for Pb are 10–100 ppm, and some extracts were further diluted for determination of Pb concentrations within the detection limits.

Statistical analysis

We used repeated-measures ANOVA to evaluate the response of plants to Pb and water stress over time. Treatment time (30, 60 and 90 days) was a within-subject factor, and Pb and water stress were fixed between-subjects factors. As the repeated-measures ANOVA showed significant effects of Pb and water stress, a separate ANOVA was used to evaluate the effects of Pb at the same water level, with Pb as fixed factor. An independent sample *t*-test was used to evaluate the effect of water stress on these parameters under the same Pb level, using water level as a grouping variable. The separate ANOVA and *t*-test were also used in analysis on biomass and Pb concentration in organs.

Before the ANOVA was conducted, data were checked for normality and homogeneity of variance, and were squared root-transformed when necessary to meet these assumptions. Multiple comparisons of means were performed using LSD tests at $\alpha = 0.05$ when ANOVA results were significant. All statistical analyses were performed with the SPSS version 13.0 (SPSS, Chicago, IL, USA), and figures were plotted with Sigmaplot 11.0 (Systat Software, San Jose, CA, USA).

RESULTS

Abundance of each bud type

Lead concentration, water stress and Pb × water had no significant effects on the abundance of axillary shoot buds, while other types of bud and total bud abundance were significantly affected by these factors and interactions with treatment time (Table 1).

In the well-watered treatment, the number of axillary rhizome buds on plants subjected to Pb treatment (500, 1500, 3000 or 4500 mg·kg^{-1}) was reduced significantly compared to controls, falling 35.89, 76.82, 82.78 and 83.97% after 60 days, and 2.60, 14.61, 43.34 and 74.03% after 90 days of treatment, respectively. The decrease in number of apical rhizome buds after 60 and 90 days of treatment was of similar magnitude. At the same Pb level, there was a significant decrease in axillary rhizome buds and apical rhizome buds of plants in the drought treatment compared to the well-watered treatment after 90 days (Table 2).

There was a significant reduction in total number of buds with the four Pb concentrations after 60 days, and at 3000 and 4500 mg·kg^{-1} Pb after 90 days in the well-watered treatment, while the reduction was always noticeably lower in Pb treatments under drought conditions as the treatment period progressed (Table 2).

Abundance of daughter shoots

We observed only emergence of daughter axillary shoots from axillary shoot buds after 30 days of treatment. The number of daughter axillary shoots, apical rhizome shoots and total abundance of daughter shoots were all significantly affected by Pb, water stress, treatment time, and their interactions (Table 1).

The presence of Pb tended to increase the number of daughter axillary shoots, although there were no significant changes after 60 and 90 days of Pb exposure in the well-watered environment, there were significant changes after 30 days of treatment (Table 3). During each treatment period, the abundance of daughter axillary shoots in the drought treatment fell significantly at Pb levels >500 mg·kg^{-1}. For a given dose of Pb, the abundance dropped significantly under drought conditions compared to the wet environment in all three measured time periods (Table 3). Most daughter shoots were daughter axillary shoots and increased with Pb concentration and duration of treatment only in well-watered plants.

In contrast to the control, Pb treatments caused a significant decline in the abundance of daughter apical rhizome shoots of 27.25, 0.12, 58.53 and 80.24% after 60 days of treatment, and 4.35, 13.50, 29.89 and 55.98% after 90 days in the well-watered treatment. In the control and under each dose of Pb, the abundance dropped significantly in the drought treatment compared to the well-watered treatment, and the reductions were larger with Pb application compared to the control after 60 and 90 days of treatment (Table 3).

As Pb concentration increased, the total abundance of daughter shoots tended to decrease, but without pronounced changes after 90 days of exposure in the moist treatment. However, there was a pronounced decrease at Pb levels >500 mg·kg^{-1} in each treatment interval under drought conditions. In the control and at each Pb level, the total number of daughter shoots was markedly lower in the drought treatment compared to in the waterlogged environment (Table 3).

Rhizome parameters

Lead, water stress, treatment time and their interactions had remarkable effects on the number of rhizomes, rhizome nodes and total rhizome length (Table 1). Pb treatments caused a significant decline in the abundance of rhizome nodes, and the reduction after 90 days was less than that after 60 days at each Pb level (except 4500 mg·kg^{-1}; Fig. 1a and b). A similar trend occurred in rhizome internode length, number of rhizomes and total rhizome length in the well-watered environ-

Table 1. Results of repeated-measures ANOVA of the effect of Pb and water stress on belowground buds, aboveground daughter shoots and rhizomes of *P. australis* after 30, 60 and 90 days of treatment.

	within subject			between subject			
parameter	Pb	W	Pb × W	T	T × Pb	T × W	T × Pb × W
buds and daughter shoots (no. plant^{-1})							
no. axillary shoot buds	0.308	0.613	0.506	<0.001	<0.01	0.51	0.418
no. axillary rhizome buds	<0.001	<0.001	<0.01	<0.001	<0.001	<0.001	<0.001
no. apical rhizome buds	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
total no. of buds	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
no. daughter axillary shoots	<0.001	<0.001	<0.001	<0.001	<0.05	<0.001	<0.001
no. daughter axillary rhizome shoots	0.209	0.201	0.512	0.264	0.515	0.060	0.064
no. daughter apical rhizome shoots	<0.001	<0.001	0.103	<0.001	<0.001	<0.001	0.061
total no. daughter shoots	<0.001	<0.001	<0.05	<0.001	0.149	<0.001	<0.01
rhizome parameters							
no. rhizomes (no. plant^{-1})	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
total rhizome length (cm. plant^{-1})	<0.001	<0.001	<0.01	<0.001	<0.001	<0.001	<0.001
no. rhizome nodes (no. plant^{-1})	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
rhizome internode length (cm. plant^{-1})	<0.001	<0.001	0.076	<0.001	<0.001	<0.001	<0.001

The number of buds, daughter shoots and rhizome parameters (number of rhizomes, total rhizome length, number of rhizome nodes and rhizome internode length) are expressed per parent shoot. W: Water stress; T: Treatment time; T×W: Interaction of treatment time and water treatment; T×Pb×W: Interaction of treatment time, Pb and water treatments.

Table 2. Number of belowground buds (axillary shoot buds, axillary rhizome buds and apical rhizome buds) of *P. australis* subjected to Pb for 30, 60 or 90 days under well-watered or drought conditions.

			Pb concentration (mg·kg^{-1})				
water level	days	bud no. (no. plant^{-1})	control	500	1500	3000	4500
WW	30	ASB	0.85 ± 0.10a	0.78 ± 0.06a	0.63 ± 0.09ab	0.68 ± 0.11ab	0.50 ± 0.04b
	60	ASB	0.90 ± 0.20b	0.65 ± 0.06b	0.76 ± 0.07b	0.68 ± 0.08b	1.29 ± 0.14a
		AxRB	1.89 ± 0.28a	1.21 ± 0.15b	0.44 ± 0.12c	0.33 ± 0.14c	0.30 ± 0.07c
		ApRB	0.57 ± 0.15a	0.36 ± 0.06ab	0.31 ± 0.07ab	0.24 ± 0.09b	0.16 ± 0.04b
		TB	3.36 ± 0.24a	2.22 ± 0.16b	1.51 ± 0.06bc	1.25 ± 0.19c	1.75 ± 0.21c
	90	ASB	0.39 ± 0.15b	0.37 ± 0.14b	0.38 ± 0.09b	0.41 ± 0.09b	0.74 ± 0.12a
		AxRB	6.16 ± 0.28a	6.00 ± 0.70a	5.26 ± 0.42a	3.49 ± 0.22b	1.60 ± 0.27c
		ApRB	3.11 ± 0.34a	3.08 ± 0.08a	2.86 ± 0.27a	2.54 ± 0.12a	1.61 ± 0.24b
		TB	9.66 ± 0.37a	9.45 ± 0.37a	8.50 ± 0.75a	6.44 ± 0.23b	3.95 ± 0.54c
D	30	ASB	0.82 ± 0.06a	0.78 ± 0.09a	0.60 ± 0.04a	0.58 ± 0.15a	0.58 ± 0.09a
	60	ASB	0.70 ± 0.04ab	0.54 ± 0.06b	0.64 ± 0.08b	0.71 ± 0.09ab	0.85 ± 0.07a*
		AxRB	1.82 ± 0.36a	1.40 ± 0.20a	0.36 ± 0.13b	0.14 ± 0.06bc	0.08 ± 0.05c*
		ApRB	0.89 ± 0.12a	0.57 ± 0.05b	0.36 ± 0.06bc	0.31 ± 0.11c	0.10 ± 0.06c
		TB	3.41 ± 0.18a	2.51 ± 0.16b	1.36 ± 0.16c	1.16 ± 0.15c	1.03 ± 0.17c*
	90	ASB	0.60 ± 0.05a	0.69 ± 0.15a	0.54 ± 0.05a	0.51 ± 0.04a	0.46 ± 0.15a
		AxRB	5.50 ± 0.51a	4.14 ± 0.35b*	2.13 ± 0.13c*	0.25 ± 0.09d*	0.31 ± 0.10d*
		ApRB	3.02 ± 0.17a	2.81 ± 0.15a	1.91 ± 0.15b*	0.72 ± 0.11c*	0.40 ± 0.07c*
		TB	9.12 ± 0.51a	7.64 ± 0.49b	4.57 ± 0.27c*	1.49 ± 0.13d*	1.17 ± 0.19d*

WW = Well-watered; D = Drought; ASB = No. axillary shoot buds; AxRB = No. axillary rhizome buds; ApRB = No. apical rhizome buds; TB = Total no. buds. The data are expressed as mean ± SE, with four replicates per treatment (n = 40). Different letters indicate significant differences ($P \leq 0.05$) between Pb levels (within one water treatment level), asterisk indicates significant difference ($P \leq 0.05$) between water treatment and drought treatment (within one Pb level).

Table 3. Number of daughter shoots (axillary shoots, axillary rhizome shoots and apical rhizome shoots) of *P. australis* subjected to Pb stress for 30, 60 or 90 days under well-watered or drought conditions.

			Pb concentration(mg·kg^{-1})				
water level	days	DSN (no. plant^{-1})	control	500	1500	3000	4500
WW	30	DAS	1.55 ± 0.06a	1.48 ± 0.13a	1.33 ± 0.06a	0.98 ± 0.09b	1.00 ± 0.14b
	60	DAS	1.87 ± 0.13a	2.22 ± 0.17a	2.17 ± 0.16a	2.19 ± 0.18a	2.14 ± 0.10a
		DAxRS	0.03 ± 0.03a	0.00 ± 0.00a	0.03 ± 0.03a	0.00 ± 0.00a	0.00 ± 0.00a
		DApRS	1.67 ± 0.10a	1.22 ± 0.13b	1.00 ± 0.10bc	0.69 ± 0.17c	0.33 ± 0.09c
		TDS	3.57 ± 0.14a	3.44 ± 0.19a	3.20 ± 0.23ab	2.88 ± 0.18b	2.47 ± 0.07b
	90	DAS	2.83 ± 0.28a	2.76 ± 0.13a	2.74 ± 0.19a	3.07 ± 0.26a	3.34 ± 0.23a
		DAxRS	0.05 ± 0.03a	0.05 ± 0.03a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
		DApRS	1.84 ± 0.11a	1.76 ± 0.23ab	1.61 ± 0.15ab	1.29 ± 0.14b	0.81 ± 0.12c
		TDS	4.72 ± 0.19a	4.57 ± 0.31a	4.35 ± 0.25a	4.36 ± 0.15a	4.15 ± 0.26a
D	30	DAS	1.05 ± 0.06a*	1.05 ± 0.10a*	1.05 ± 0.06a*	0.73 ± 0.09b*	0.40 ± 0.07c*
	60	DAS	1.67 ± 0.19a	1.36 ± 0.10a*	1.31 ± 0.13ab*	0.98 ± 0.06b*	0.95 ± 0.13b*
		DAxRS	0.00 ± 0.00a	0.03 ± 0.03a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
		DApRS	0.77 ± 0.06a*	0.60 ± 0.05a*	0.20 ± 0.07b*	0.19 ± 0.03b*	0.00 ± 0.00c*
		TDS	2.44 ± 0.19a*	1.99 ± 0.09a*	1.51 ± 0.11b*	1.17 ± 0.06bc*	0.95 ± 0.13c*
	90	DAS	1.65 ± 0.09a*	1.86 ± 0.11a*	1.25 ± 0.09b*	1.23 ± 0.06bc*	0.97 ± 0.09c*
		DAxRS	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
		DApRS	0.98 ± 0.14a*	0.81 ± 0.11a*	0.88 ± 0.11a*	0.20 ± 0.07b*	0.06 ± 0.04b*
		TDS	2.63 ± 0.17a*	2.67 ± 0.05a*	2.13 ± 0.11b*	1.43 ± 0.10c*	1.03 ± 0.06d*

WW = Well-watered; D = Drought; DSN = No. daughter shoots; DAS = No. daughter axillary shoots; DAxRS = No. daughter axillary rhizome shoots; DApRS = No. daughter apical rhizome shoots; TDS = Total no. daughter shoots. The data are expressed as mean ± SE, with four replicates per treatment (n = 40). Different letters indicate significant differences ($P \leq 0.05$) between Pb levels (within one water treatment level), asterisk indicates significant difference ($P \leq 0.05$) between water treatment and drought treatment (within one Pb level).

ment (Fig. 1c–h). The reduction in rhizome parameters, in order, was total rhizome length >rhizome or rhizome node number >rhizome internode length at the same Pb level compared to controls after 90 days in both water treatments (Fig. 1). In the control and at any dose of Pb, rhizome internode length, number of rhizomes and total rhizome length

decreased significantly in droughted plants compared to well-watered plants after 60 and 90 days, and the reduction in all three parameters after Pb treatments were higher than in the controls (Fig. 1c–h).

Biomasses of organs

Aboveground shoot biomass was significantly inhibited at Pb levels of 3000 and 4500 mg·kg^{-1} under well-watered condition, and Pb > 500 mg·kg^{-1} under drought condition (Fig. 2a). In controls and at each Pb level aboveground shoot biomass in the drought treatment was significantly less than that in well-watered treatment (Fig. 2a). Similar responses occurred in rhizome and root biomass (Fig. 2b and c).

Lead concentrations in organs

Lead concentrations in organs were positively correlated with the number of axillary shoot buds in the well-watered treatment, but showed a negative relationship with growth of other clonal propagative modules in both water treatments (Table 4). The Pb concentrations in organs decreased in the order roots >shoots >rhizomes, and in roots or rhizomes were higher in the well-watered treatment than in the drought treatment at each Pb level (Fig. 3). The inverse responses occurred in shoots at 3000 and 4500 mg Pb·kg^{-1}.

DISCUSSION

Clonal module growth and propagation

Our study showed that daughter axillary shoots contributed more to *P. australis* reproduction than daughter rhizome shoots that emerged from rhizome buds, especially after Pb exposure. We suggest three possible reasons for these findings. First, it might be easier for the axillary shoot buds attached to the basal part of stems to emerge from a shallow layer of soil, rather than grow horizontally to develop into main rhizomes.

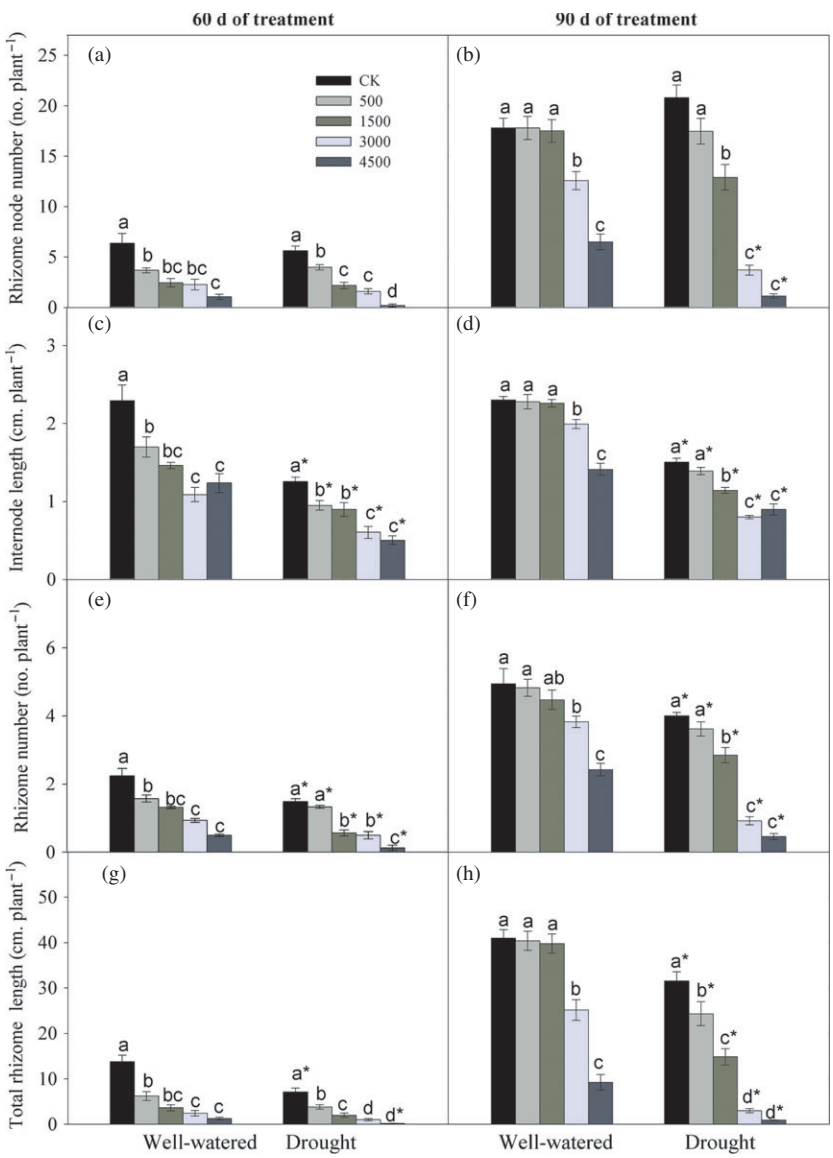

Fig. 1. The growth of rhizomes of *Phragmites australis* exposed to Pb stress after 60 and 90 days of treatment under well-watered and drought conditions; a, b: Number of rhizome nodes; c, d: Rhizome internode length; e, f: Number of rhizomes; g, h: Total rhizome length; Different letters indicate significant differences ($P \leq 0.05$) between Pb levels (within one water treatment level), and an asterisk indicates a significant difference between water treatment and drought treatment (within one Pb level).

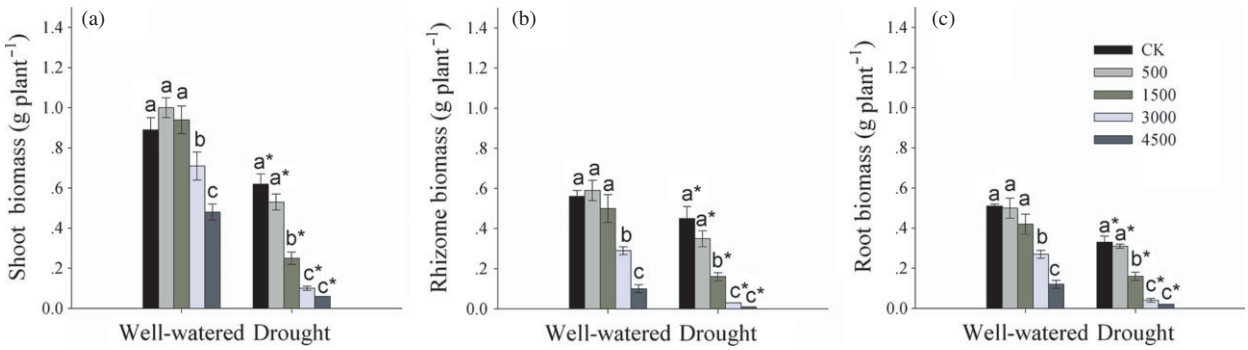

Fig. 2. Shoot, rhizome and root biomasses of *Phragmites australis* exposed to Pb stress after 90 days of treatment under well-watered and drought conditions. Different letters indicate significant differences ($P \leq 0.05$) between Pb levels (within one water treatment level), and an asterisk indicates a significant difference ($P \leq 0.05$) between water treatment and drought treatment (within one Pb level).

Table 4. Pearson correlation analysis of Pb concentrations in organs and growth of clonal propagative modules (no. of buds or daughter shoots and rhizome growth parameters) after 90 days of Pb exposure (R represents the Pearson correlation coefficient).

	R^2											
	no. of daughter shoots			no. of buds				rhizome				
Pb concentrations in organs (mg kg^{-1})	axillary shoot	apical rhizome shoots	total	axillary shoot bud	axillary Rhizome bud	apical Rhizome bud	total	root length	length	node no.	internode length	rhizome no.
well-watered												
root	0.43^{ns}	−0.79**	−0.41^{ns}	0.48*	−0.89**	−0.77**	−0.88**	−0.94**	−0.86**	−0.84**	−0.88**	−0.90**
rhizome	0.48*	−0.79**	−0.36^{ns}	0.53*	−0.90**	−0.78**	−0.88**	−0.95**	−0.89**	−0.87**	−0.90**	−0.90**
shoot	0.46*	−0.81**	−0.40^{ns}	0.47*	−0.90**	−0.79**	−0.90**	−0.95**	−0.90**	−0.87**	−0.89**	−0.91**
drought												
root	−0.77**	−0.83*	−0.91**	−0.29^{ns}	−0.84**	−0.91**	−0.87**	−0.96**	−0.88**	−0.91**	−0.83**	−0.92**
rhizome	−0.78**	−0.88**	−0.95**	−0.35^{ns}	−0.86**	−0.93**	−0.90**	−0.97**	−0.90**	−0.93**	−0.83**	−0.95**
shoot	−0.74**	−0.81**	−0.89**	−0.30^{ns}	−0.76**	−0.86**	−0.80**	−0.93**	−0.82**	−0.86**	−0.72**	−0.88**

*$P < 0.05$; **$P < 0.01$; $^{ns}P > 0.05$ (two tailed).

Second, the energy investment in axillary buds on rhizomes from the deep soil layer is lower because their high rate of respiration would deplete much of the available energy under anoxic conditions (Klimeš *et al.* 1993; Hendrickson & Briske 1997) according to the cost–benefit hypothesis (Vesk & Westoby 2004). Third, rhizome buds in deeper soil layers are fully exposed to Pb, potentially leading to Pb toxicity to plant development and resprouting capacity. Moreover, daughter apical rhizome shoots were produced from apical rhizome buds, but no daughter axillary rhizome shoots emerged from axillary rhizome buds at either water treatment (Tables 2 and 3), similar to the findings of previous research (Yang & Lang 1998). This phenomenon might be related to the biological characteristics of *P. australis*; growth space must first be expanded before the number of daughter rhizome shoots can increase (Mook & Van der Toorn 1982). Another explanation could be that the limited resources that axillary buds require for active growth are usurped by apical dominance (Phillips 1975), such that the axillary rhizome buds cannot compete successfully for resources against the apical rhizome buds, which are still stronger when nutrients or water are scarce (McIntyre 1969, 1976). Therefore, once axillary rhizome buds are produced after 60 days of treatment, they would tend to continue horizontal growth to develop into secondary rhizomes, particularly in the well-watered treatment, leading to a relatively high increase in total number of rhizomes that extend to obtain resources for production of new apical rhizome buds.

The ability of plants to survive disturbances is related to either a tolerance or an escape strategy (Briske 1996). Howard & Rafferty (2006) demonstrated that *P. australis* acquires stress tolerance through clonal variations in growth. As the treatment period progressed, the development and upward output of axillary shoot buds that was stimulated by Pb caused an increase in the number of daughter axillary shoots, in contrast to controls, especially at 3000 and 4500 mg Pb·kg^{-1} in the late period of the well-watered treatment (Tables 2–4) — this can be considered as a tolerance strategy (Turnbull *et al.* 1997; Mony *et al.* 2011). Simultaneously, the increase in abundance of daughter axillary shoots would increase available photosynthetic area, which would favour plant growth. In contrast, Pb concentrations above 3000 mg·kg^{-1} in the soil inhibited the formation of axillary and apical rhizome buds, and restrained the growth of apical rhizome buds into daughter apical rhizome shoots late during the well-watered treatment (Tables 2 and 3) — combined, these results indicate an escape strategy. In addition, development and activity of belowground buds is

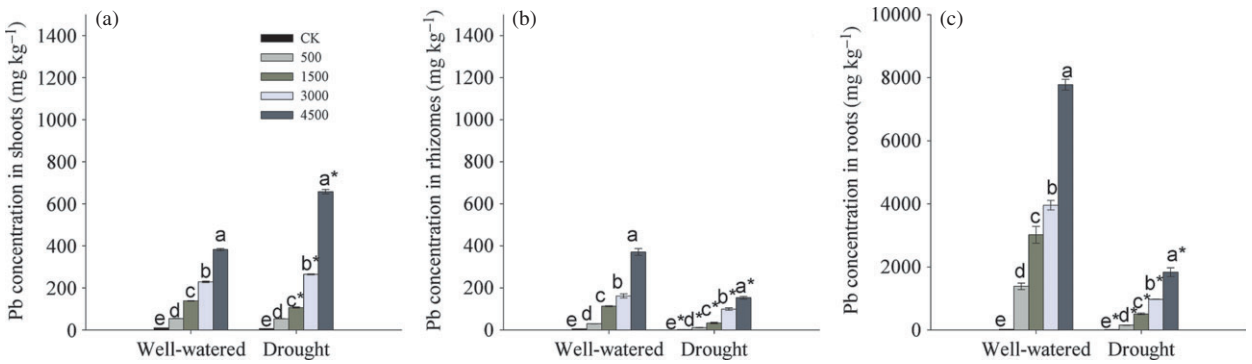

Fig. 3. Pb concentrations in organs of *Phragmites australis* exposed to Pb stress after 90 days of treatment under well-watered and drought conditions. Different letters indicate significant differences ($P \leq 0.05$) between Pb levels (within one water treatment level), and an asterisk indicates a significant difference ($P \leq 0.05$) between water treatment and drought treatment (within one Pb level).

influenced by N availability (McIntyre 1972a,b). High N supply promotes shoot development from rhizome buds to a greater extent than rhizome development, and their development prefers NH_4^+-N (McIntyre1972b, 1987; McIntyre & Cessna 1998). The root or/and rhizome buds have an advantage over axillary shoot buds in competing for increased N supply (Myers *et al.* 1964; Briske 1991). Similarly, more N is accumulated in belowground than in aboveground parts of *P. australis*, with lowest levels in stems (Zeng & Zhang 2009). However, the amount of N exported to the shoots was only sufficient for growth of the main shoot apex and early development of axillary shoot buds (McIntyre 1972a). Therefore, in our research, the fall in number of rhizome buds might be caused by the inhibitory effect of Pb addition without N stimulation. Conversely, the increasing number of axillary shoot buds might be correlated with NO_3^--N export to the shoots and its effective utilisation, because *P. australis* may benefit from NO_3^- in shallow water or even in terrestrial stands, although wetland plant species generally prefer NH_4^+ rather than NO_3^- (Munzarovaa *et al.* 2006). Further investigations are required to determine the effects of Pb contamination, various forms of N and their interaction on growth and development of different types of bud of *P. australis* grown in contrasting habitats.

The axillary rhizome buds, apical rhizome buds, daughter apical rhizome shoots and rhizome parameters (number of rhizomes and nodes, length of rhizomes and internodes) showed similar trends in the well-watered environment, *i.e.* at each Pb (<4500 mg·kg^{-1}) level, the reductions after 90 days were significantly less than after 60 day (Tables 2 and 3, Fig. 1). This pattern can be explained by the 'compensation' theory of Ruttkay *et al.* (1964): more active plant growth would occur during the later period in order to make up for the loss caused by disturbance in early stages. Daughter rhizome shoots contribute more to plant population expansion, while axillary daughter shoots contribute more to plant population maintenance (Wang *et al.* 2008; Bai *et al.* 2009). Clearly, the compensation created by the number of daughter apical rhizome shoots and the increased number of daughter axillary shoots produced no changes in the total number of daughter shoots, creating a timely supplement to the aboveground population density of reeds subjected to Pb in this study. Further, the compensation in abundance of axillary and apical rhizome buds during the late period of treatment would prepare for development and elongation of rhizomes and population recruitment and expansion during the following year, outcomes that would favour clonal propagation and population stability of *P. australis* subjected to Pb stress in wet environments in the long term. Nevertheless, the abundance of each type of bud or daughter shoot was reduced significantly by the synergistic effects of drought and Pb, resulting in a remarkable decrease in total abundance of buds and daughter shoots at Pb >500 mg·kg^{-1} under drought conditions (Tables 1–3). These results imply that the reduced clonal propagative capacity would decrease population density and survival of *P. australis* grown under higher Pb contamination and drought.

The belowground vegetative propagation organs (*e.g.* rhizomes) of herbaceous plants may lead to clonality (Klimešová & Klimeš 2007), *i.e.* when a set of daughter shoots are connected to each other *via* rhizomes, the lateral spread of rhizomes ensures that daughter shoots are placed in an environment that is similar to conditions in which the mother plant is able to reproduce (Klimešová & Klimeš 2008). It is clear that perennial rhizome-bearing buds, as a progenitive fragment or structure, plays a crucial role in the clonal propagation of *P. australis* and the elongation or spread is useful as an indicator to assess clonal reproduction. The elongation of rhizomes is determined by four rhizome parameters, rhizome number, rhizome length, rhizome node number and rhizome internode length. In this study, the reduced total rhizome length was mainly attributable to reduced abundance of rhizomes and rhizome internodes, because the adverse effect of Pb was least on rhizome internode length (Fig. 1). The lateral spread of rhizomes may expand spatial occupation (Prach & Pyšek 1994; Henry & Amoros 1996; Barsoum 2002) to provide access to resources that could be mobilised for regrowth of daughter shoots from buds stored below the ground (Barrat-Segretain & Amoros 1996; Brewer & Bertness 1996; Henry & Amoros 1996), thereby maintaining clonal propagation and population expansion of plants after disturbance. Some authors have argued that the belowground organs of perennial herbs conserve carbon resources and buds for spring regeneration and resprouting after disturbance (Klimešová & Klimeš 2003, 2007). Thus, the compensatory growth of rhizome parameters at Pb ≤ 1500 mg·kg^{-1} in the late period promoted the spread of rhizomes and their absorption of resources, together with development and resprouting of buds (*i.e.* clonal

reproduction) in the well-watered environment. However, the spatial colonisation through rhizome elongation was still significantly inhibited at Pb levels of 3000 and 4500 mg·kg^{-1}. On the other hand, the short average length of rhizomes (calculated as the ratio of total rhizome length to rhizome number) might result in short daughter apical rhizome shoots with a small distance between them, *i.e.* a clumped growth form (Clevering 1999). This phenomenon could be an adaptive growth response to high Pb contamination in a moist environment. In addition, water deficiency had a synergistic interaction with Pb, so that declines in the four rhizome parameters occurred in the dry environment (Fig. 1). In short, the tolerance strategies of daughter axillary shoots, and compensatory growth of buds, daughter shoots and rhizomes, enhanced the clonal propagative resistance of *P. australis* to the presence of Pb (except at 4500 mg Pb·kg^{-1}), which would enable populations to spread under well-watered conditions. These two strategies can be considered as an adaptive growth mechanism, *i.e.* the rhizomes and different types of buds and daughter shoots have the flexibility to adapt to Pb contamination through the two strategies in waterlogged environments. Conversely, the synergistic effect of drought and Pb (above 500 mg Pb·kg^{-1}) on rhizome parameters, *e.g.* buds and daughter shoots, significantly inhibited clonal modular growth and reproduction in the dry environment.

From above results, the growth and propagation of clonal modules was inhibited significantly by Pb of 4500 mg·kg^{-1} in well-watered conditions and by Pb levels of >500 mg·kg^{-1} in the drought treatment. A similar response occurred in biomass of plant organs (Fig. 2). We speculate that Pb concentrations of 4500 and 500 mg·kg^{-1} in soil might meet or exceed the Pb tolerance threshold of clonal propagation of *P. australis* in well-watered and drought environments, respectively.

Biomass and Pb concentration in organs

Our results show that in both wet and dry conditions Pb was restrained when transporting into shoots (Fig. 3). This is supported in findings of previous researches (Weis *et al.* 2004; Bonanno & Giudice 2010), and similar accumulations of other metals have also been observed in *P. australis* (Ye *et al.* 2003; Hechmi *et al.* 2014). The filtering effect of roots is the most effective strategy for protecting rhizomes and shoots (Sneller *et al.* 1999). However, the high Pb concentrations in roots inhibited root growth, reducing the proportion of root biomass in total biomass in treatments of 3000 and 4500 mg Pb·kg^{-1} in particular (Fig. 2c). In addition, we found that the lowest Pb accumulation occurred in rhizomes (Fig. 3b), as also found by Fürtig *et al.* (1999), where values were lower in rhizomes compared to that in shoots. This might be because the rhizome is involved in transport of heavy metals, and direct ion uptake through the outer cork layer is impossible (Fürtig *et al.* 1999; Bankó *et al.* 2002). Moreover, many papers have found that translocation of Pb from the rhizome to the shoot is low (Ye *et al.* 1997; Vymazal *et al.* 2007). Malkowski *et al.* (2002) suggested that Pb accumulated in roots might move to shoots *via* induction from an unknown signal. Therefore, we speculated that the higher Pb concentration in shoots might be due to translocation from roots, not rhizomes, because rhizomes might be mainly involved in Pb transport rather than accumulation. As a typical clonal plant, *P. australis* relies on vegetative reproduction of rhizomes, which act as reproductive fragments, bearing buds that enable expansion into new spaces (Hara *et al.* 1993; Klimeš & Klimešová 2000). It is clear that allocation of the least Pb to rhizomes could constitute a strategy that protects the development and/or output of buds from Pb toxicity in order to maintain population stability or expansion in environments with heavy Pb contamination. At soil Pb levels of 3000 and 4500 mg·kg^{-1}, the higher Pb concentration in shoots under dry rather than wet conditions might be more detrimental to reed growth because aboveground shoots are the main photosynthetic and metabolic organs. As a result, the biomass of organs was inhibited significantly in the drought environment (Fig. 2). At each level of Pb treatment, the Pb concentration in rhizomes and roots was higher in well-watered *versus* drought conditions (Fig. 3), but growth of clonal propagative modules was better in the moist treatment, *e.g.* higher biomass and number of buds, daughter shoots and rhizomes and longer rhizome length (Tables 2 and 3, Figs 1 and 2). These findings suggest that reeds grown under Pb contamination and moist conditions show higher tolerance and adaptation to Pb than those in the droughted environment. Ye *et al.* (1998) found that reeds grew better in fly ash in flooded rather than under dry conditions, but the reverse trend occurred when grown in mine tailings. This inconsistency might be due to the various kinds and levels of heavy metals in the different growth media.

CONCLUSIONS

Clonal propagative resistance to Pb contamination may be conferred through the tolerance strategy of daughter axillary shoots, and compensatory growth of axillary shoot buds, apical rhizome buds, daughter apical rhizome shoots and rhizomes, and the strategy of Pb allocation in organs, all of which facilitate population recruitment of *P. australis* in wet environments. However, clonal module growth and reproductive ability were inhibited significantly by the synergic effect of drought and Pb, which cause *P. australis* populations in drought environments to decline. In addition, soil Pb concentrations of 4500 and 500 mg·kg^{-1} might meet or exceed the Pb tolerance of clonal propagative ability in wet and dry environments, respectively.

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