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Table: 2, Figs : 7

Allelopathic potential of berberine from *Coptis chinensis* Franch on invasive weeds

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ABSTRACT

Berberine significantly inhibited the root growth of several invasive weeds [*Bidens pilosa* L., *Mikania micrantha* Kunth, *Conyza bonariensis* (L.) Cronq., *Pistia stratiotes* L., *Alternanthera philoxeroides* Griseb and the the plant *Arabidopsis thaliana*]. The IC_{50} value of berberine after 7-days, on the fresh weight of *B. pilosa* was lower than the IC_{50} value of glyphosate or pendimethalin herbicides. In pot experiments, berberine decreased the fresh weight of roots and stems of *B. pilosa*. In *A. thaliana* transgenic CYCB1: GUS line, the berberine decreased the CYCB1 activity *and* inhibited the cell division in the roots. These results suggested that berberine can be used as a biocontrol agent to control the invasive weeds.

Key words: Allelopathic potential, *Alternanthera philoxeroides*, *Arabidopsis thaliana*, berberine, *Bidens pilosa*, biological control, *Conyza bonariensis*, *Coptis chinensis*, dry weight, fresh weight, invasive weed, *Mikania micrantha*, *Pistia stratiotes*.

INTRODUCTION

Biological invasions of the exotic species worldwide contribute to global environmental change (15) and invasion, is most serious threat to native genes, biodiversity and ecosystem function and causes huge economic losses (4,11). Owing to the robust vitality and stubborn invasiveness of exotic plants, providing a satisfactory control is a great challenge, currently, these are controlled by synthetic herbicides. However, the indiscriminate use of synthetic chemicals has resulted in various toxicological effects to the environment. In the past few decades, there has been an increased global interest in using plant based chemicals with reduced health and ecological hazards as alternative to chemicals control (3). Thus, native plant species that act as antagonistic to exotic invasive species have generated increasing interest (29,42).

Bidens pilosa L. *Mikania micrantha* Kunth and *Conyza bonariensis* (L.) Cronq. are terrestrial invasive weeds in crop fields and in forests (including the *Eucalyptus* forest) in many Asian and European countries (24,31,35,43). while *Pistia stratiotes* L. and *Alternanthera philoxeroides* Griseb are aquatic invasive weeds in many Asian, Europian and American countries (5-7,9,28,37). Invasive plants introduced unintentionally through agricultural or intentionally for ornamental purposes, have become major weeds in crops and threat to native fauna and ecological systems.

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The extracts of *Coptis chinensis* Franch (Chinese goldthread) from central China, significantly inhibited the root growth of the invasive weed, *P. stratiotes* (38,47). Its rhizomes are used in traditional Chinese medicine and are source for isoquinoline alkaloids berberine, palmatine, hydrastine and coptisine (19,41). Berberine is an isoquinoline alkaloid found in the roots, stem and bark of *Berberis* plant. It is widely distributed in several botanical families, such as Berberidaceae, Papaveraceae, Fumaraceae, and Menispermaceae. It is also a powerful herbal product used as an immune system booster, antioxidant to treat intestinal and lung conditions, skin diseases and various other illnesses (2,10,17,20,21,46). Generally, berberine is considered as non-toxic alkaloid at doses used in clinical situations (18,32). Therefore, the use of berberine as safe and soil applied herbicide to control the invasive plants is promising. This study aimed to explore the potential of *C. chinensis* extract (contains berberine) to control the invasive weeds, *B. pilosa*, *M. micrantha*, *C. bonariensis*, *P. stratiotes* and *A. philoxeroides*.

METHODS AND MATERIALS

I. Isolation and identification of Berberine

Rhizomes of *C. chinensis* were collected during July, 2010 from Lichuan County, Hubei Province, China. The herbarium specimen was authenticated by Prof. Bingtao Li, College of Forestry, South China Agricultural University. A voucher specimen was deposited at the Key Lab. of Natural Pesticides & Chemical Biology, Ministry of Education, South China Agricultural University.

Extraction and Isolation: The dried and milled rhizomes of *C. chinensis* (25 kg) were extracted with methanol (MeOH) (3 × 100 L × 24 h) at room temperature to give a residue (1100 g) after evaporating under vacuum at 50 °C. This residue was suspended in H_2O (3000 mL) and then extracted successively with petroleum ether (3 × 4 L), EtOAc (3 × 4L), and n-BuOH (3 × 4 L) to give a petroleum ether-soluble portion (160 g), an EtOAc-soluble portion (330 g), and an n-BuOH-soluble portion (210 g). The EtOAc-soluble portion (330 g) was chromatographed over a silica gel column (900 g) and eluted with $CHCl_3$-MeOH [100:0 (1.5L), 50:1 (2 L), 20:1 (8 L), 10:1 (10 L), 0:100 (1.5L)]. Gradient elution with $CHCl_3$-MeOH (80:20) gave fractions B (8 g). Fraction B was purified on Sephadex LH-20 (150g, MeOH, 3 L) to afford yellow crystals C (265 mg). TLC was performed on precoated TLC plates (200-250 μm thickness, F_{254} Si gel 60 and F_{254} RP-18 Si gel 60, Qingdao Marine Chemical, Inc.) with compounds visualized under UV light at 254 nm or by spraying the dried plates with 10% aqueous H_2SO_4 followed by heating until dryness. ^1H and ^{13}C NMR spectra were measured on Bruker AV-400 and DRX-500 instruments (Bruker, Zurich, Switzerland) using TMS as internal standard for chemical shifts. Chemical shifts (δ) were expressed in ppm with reference to the TMS resonance (45). We used this process to isolate the major component, berberine and did not isolated other compounds.

II. Bioassays

(i). Crude extract and Berberine on *B. pilosa, C. bonariensis* and *M. micrantha*

Preliminary experiments were done to evaluate the effects of methanol extract of *C. chinensis* on the growth of *B. pilosa*, *C. bonariensis* and *M. micrantha*. The results showed that the effects of solvent were negligible at < 1% (MeOH) concentration. Germination rate experiment revealed that the germination rates of *B. pilosa*, *C. bonariensis* and *M. micrantha* were 98%, 92% and 90%, respectively.

The herbicidal activity of crude extract of *C. chinensis* and berberine was evaluated using seeds of *B. pilosa*, *C. bonariensis* and *M. micrantha* seeds collected in September 2010 from the South China Agricultural University Campus, Guangzhou, China. Empty and undeveloped seeds were discarded by floating in tap water. The seeds were surface sterilized by treating with 15% sodium hypochlorite for 20 min and rinsed with distilled water.

The rizhome extract of *C. chinensis* was dissolved in 1% MeOH (1%, v/v), and then diluted to the desired concentrations with distilled water. Standard berberine was from Sigma-Aldrich, purity: 97%, was dissolved in distilled water, and then diluted to the desired concentrations by distilled water. The crude extract of *C. chinesis* concentrations used were: 18.75, 37.5, 75, 150 and 300μg/mL.

For germination studies, one sheet of filter paper was placed in bottom of 50 mL beaker and then moistened with 4 mL test solutions. Then, 20 seeds were placed on the filter paper with two layers of glass beads beneath in the beaker. The beakers were sealed with polyethylene films with several little holes for ventilation and incubated at 25±2°C under a photoperiod of 12 h light/12 h dark. Germination was defined as the stage when the radicle had extended more than 1 mm beyond the seed coat.

In controls for herbicidal activity, test of the extract of rizhomes, same volume of 1% MeOH-contained water (1%, v/v) was added. To the controls in the herbicidal activity test of berberine, the same volume of water was added. The beakers were then placed at 25±2^{o}C in a growth chamber (L:D=12:12, RH=75±5%). The primary root lengths were measured after 7 days. The treatments were arranged in a completely random design with three replicates. Root inhibition was calculated as under (33,36):

$$\text{Root inhibition (\%)} \quad \frac{\text{Root length of control-Root length of treatment}}{\text{Root length of control}} \times 100\%$$

(ii). Crude extract and Berberine on *A. philoxeroides* and *P. stratiotes*

Fresh samples of *A. philoxeroides* and *P. stratiotes* were collected from the uncontaminated ponds in Guangzhou, China. Plants were cultured in clean plastic containers in the laboratory for 60 d. For the bioassay of *A. philoxeroides*, the leaves and buds of selected healthy stems were cut with 2 internodes and these were cultured

in 1:40 (v/v) Hoagland's Nutrients solution (39) in transparent plastic cups. The crude extract or berberine were dissolved in hot water, diluted with distilled water and then 50 ml solution was added to each cup (100 ml in volume, 5 cm in dia). The crude extract of *C. chinesis* concentrations used were: 31.25, 62.5, 125, 250 and 500 µg/mL. The cups were sealed with polyethylene films with several little holes for ventilation and incubated at 25±2°C under a photoperiod of 12 h light/12 h dark. The controls were treated with only nutrient solution. The number of new roots were counted 15 d after incubation.

For the bioassay of *P. stratiotes*, plants with 2-3 leaves were selected. The other procedures were similar to *A. philoxeroides* bioassay. The fresh weights were measured 15 d after incubation (38,47). The treatments were replicated five times in a completely random design

(iii). *B. pilosa* bioassay with Berberine, glyphosate and pendimethalin

Berberine efficacy was tested against two herbicides (glyphosate and pendimethalin, these inhibits the root development). Berberine was dissolved in distilled water to get 2 mg/mL stock solution, glyphosate herbicide purity: 95.5%) was dissolved in water to get 5 mg/mL stock solution, pendimethalin herbicide purity: 95.2%) was dissolved in 95% alcohol to get 5 mg/mL stock solution. The solutions of these chemicals were respectively added to 13×13 (cm) MS (Murashige & Skoog) agar (40) plates to get final concentrations of 2, 4, 8, 16 and 32 µg/mL.

Achenes of *B. pilosa*, *C. bonariensis* and *M. micrantha* were surface sterilized as described above. The sterilized seeds were sown in MS agar plates. The controls were treated with sterilized water, while for pendimethalin the plates contained 0.68% alcohol (the same concentration as in the treatment). Ten seeds were placed on the plates and incubated at 25±2°C, photoperiod of 12 h light/12 h dark. The germination (%) was recorded 3 d after incubation. The fresh weight was determined 7 d after incubation (33,36). The treatments were replicated five times in a completely random design.

(iv). Effects of Berberine on maize

Seeds of maize were surface sterilized and soaked for 1 h in distilled water. Fungal and bacterial control was accomplished by treating the weed seeds with 1.0% (v/v) sodium hypochlorite for 10 min. Berberine was applied in distilled water at concentrations from 2 to 32 µg/ml. Ten seeds were placed on moistened filter paper to allow for germination, the filter paper was moistened with different berberine concentrations in a 50 ml glass beaker containing 4 ml herbicide. Similarly, a set of twenty seeds were placed on tap water moistened filter paper to serve as a control. The glass beakers were then sealed with polyethylene wrapping films with several little holes for ventilation and incubated at 25±2°C under a photoperiod of 12 hour light / 12 hour darkness. A completely randomized design was selected and all treatments were replicated at least three times. The primary fresh weight were measured at 7 days after the treatment.

(v). Effects of Berberine on *A. thaliana*

A. thaliana (CYCB1:GUS) seeds (Courtsey Prof. L.J. Feldman, Department of Plant and Microbial Biology, College of Natural Resources, University of California, Berkeley, USA**)** were surface sterilized with 10% hydrogen peroxide for 30 min and rinsed thrice with sterile water. The sterilized seeds were planted in 13×13 cms agar plates containing half-strength Hoagland's nutrients solution and berberine (5 µg/mL)**.** The plates were incubated at 25±2°C and photoperiod of 12 h light/12 h dark. Fresh weight of *A. thaliana* was determined 7 d after incubation.

For histochemical analysis of GUS (beta-glucuronidase) expression, after the treatment, roots were vacuum infiltrated, then incubated with a GUS substrate buffer (1mM 5-bromo-4-chloro-3-indolylglucuronide, 100 mM Tris, pH 7.0, 50 mM NaCl, 0.06% Triton X-100, and 0.5 mM potassium ferrocyanide) at 37°C for 3 h. The reaction was terminated by adding 70% ethanol. Root samples were visualized by DIC optics microscopy (12,44). The treatments were replicated five times in completely random design.

VI. Pot experiment

To study the effects of berberine on *B. pilosa* the pot experiment was conducted in laboratory (22 - 25 °C and relative humidity : 75±10 %). Each PVC pot (12 cm dia) was filled with 120 g air-dried sandy soil and berberine was applied at 50,100 and 200 µg/g (based on our earlier studies). The seed germination, fresh weight of roots and stems were recorded at 60 d after sowing**.**

VII. Statistical analysis

The data from root inhibition bioassays were collected from three independent repeats, with at least three replicates in each repeat. Statistical analysis was done with SPSS. The data followed by the same letter were not significantly different at P = 0.05.

RESULTS AND DISCUSSION

I. Herbicidal activity of *Coptis chinesis*

Plants are the most important bioresources of natural herbicides. Recently Lamberth (23) reported the presence of some naturally occurring amino acid derivatives with herbicidal activity. In the present study, the crude extract of *Coptis chinesis* rhizomes at 300 µg/mL, inhibited the root length of *B. pilosa*, *M. micrantha* and *C. bonariensis* by 89.12%, 96.20% and 97.97%, respectively. At 500 µg/mL, the fresh weight of *P. stratiotes* (with 2-3 leaves) and *A. philoxeroides* was reduced by 94.56% and 91.77%, respectively 15 d after the treatment (Figure 1A). Thus the crude extract of *C. chinesis* rhizomes exhibited a broad-spectrum of inhibitory activity on roots of important invasive weeds [*P. stratiotes*, *A. philoxeroides*, *C. bonariensis*, *M. micrantha*, and *B. pilosa*]. It was thus, meaningful to explore the exact active ingredient of the Chinese gold thread rhizome extract that was responsible for inhibition of these weeds.

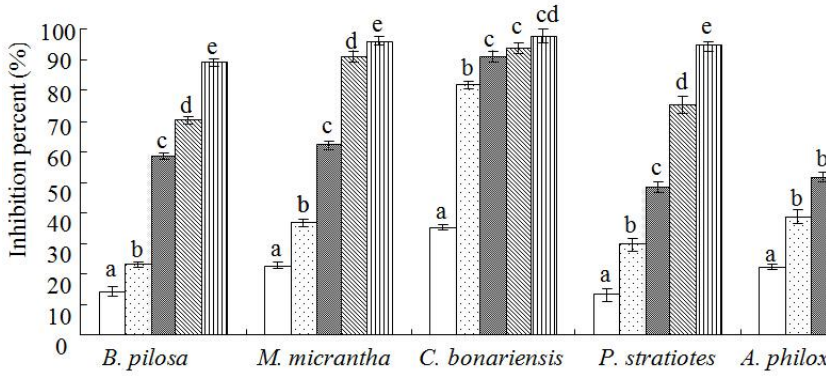


Figure 1. Effect of aqueous extract of *Coptis chinesis* on *B. pilosa*, *M. micrantha*, *C. bonariensis*, *P. stratiotes* (with 2-3 leaves) *and A. philoxeroides*. Inhibition (%) of primary root length (7d) was used for *B. pilosa*, *M. micrantha* and *C. bonariensis* was determined at 18.75, 37.5, 75, 150 and 300 µg/mL concentrations (left to right); Inhibition (%) of fresh weight (15d) was used for *P. stratiotes* (with 2-3 leaves) *and A. philoxeroides* was determined at 31.25, 62.5, 125, 250 and 500 µg/mL concentrations (left to right).

II. Fractionation of Rhizome extract and purification of berberine

By TLC and mass spectrography, the major component in the crude extract and needle-like yellow crystals in methanol was identified as berberine EI-MS m/z: 336; Chemical formula: $C_{20}H_{18}NO_4$. The brief explanation of Fig 2 and 3 is given below:

13C-NMR (CD_3OD, 150 MHz) δ: 152.3 (C-4), 152.2 (C-11), 150.1 (C-5), 146.5 (C-10), 145.9 (C-12), 139.8 (C-16), 135.3 (C-17), 132.0 (C-13). 128.3 (C-7), 124.7 (C-8), 123.5 (C-14), 122.0 (C-9), 121.7 (C-18), 109.5 (C-3), 106.7 (C-6), 103.8 (C-15), 62.7 (11-OCH_3), 57.8 (C-1), 57.4 (10-OCH_3), 28.4 (C-2)

^{1}H-NMR (CD_3OD, 600 MHz) δ: 9.77 (1H, s, H-7), 8.70 (1H, s, H-12), 8.12 (1H, d, *J* = 6 Hz, H-8), 8.00 (1H, d, *J* = 12 Hz, H-9), 7.65 (1H, s, H-6), 6.96 (1H, s, H-3), 6.11(2H, s, -O-CH_2-O-), 4.93 (2H, t, *J* = 12 Hz, H-1), 4.21 (3H, s 10-OCH_3), 4.11 (3H, s, 11- OCH_3), 3.26 (2H, t, *J* = 12 Hz, H-2).

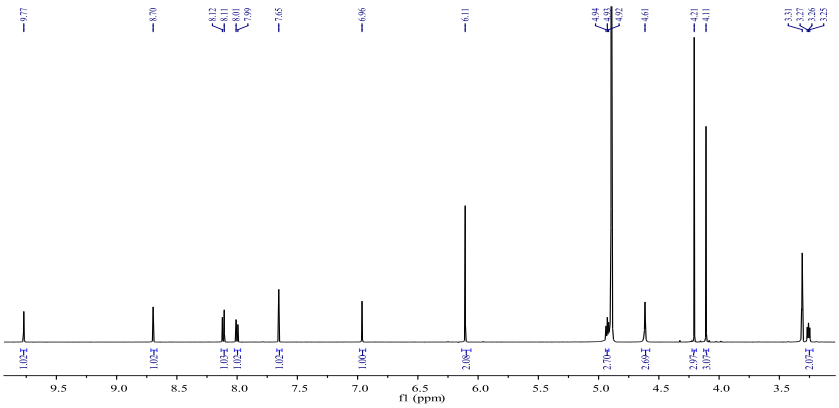


Figure 2. ^{1}H of bererine (MeOD, 600MHz) (June, 2012)

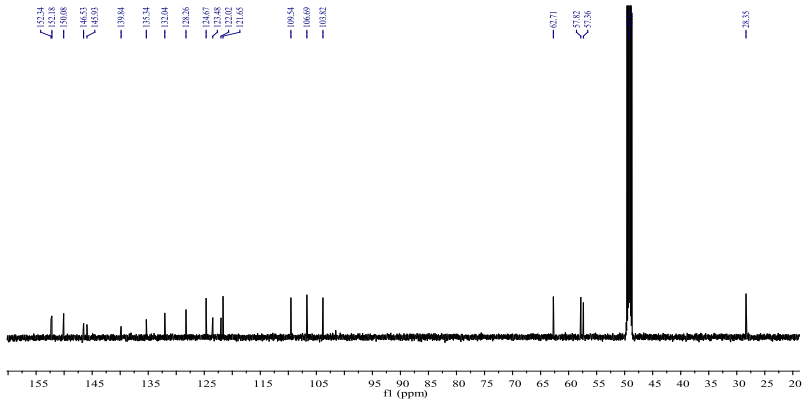


Figure 3. ^{13}C of bererine (MeOD, 150MHz) (June, 2012)

The above data were consistent with the reference standard of berberine reported by Yu and Yang (46) and Shu (34). The structure is shown in Figure 4.

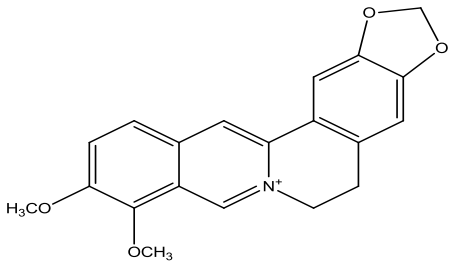


Figure 4. Molecular structure of berberine.

III. Berberine bioassay

To establish that berberine in the *Coptis chinesis* rhizomes was responsible for the growth inhibition of test weeds, We used pure berberine (97%) to assess berberine efficacy on inhibition of weed growth. The inhibitory effects of berberine on root length of test Weed spp. was determined 7 days after the treatment. Berberine at 10, 40, 25 and 0.2 μg/mL inhibited the root length of *B. pilosa (*70.84%), *M. micrantha* (99.29%), *C. bonariensis* (93.71%) and *A. thaliana* (54.03%) respectively (Fig. 5A).

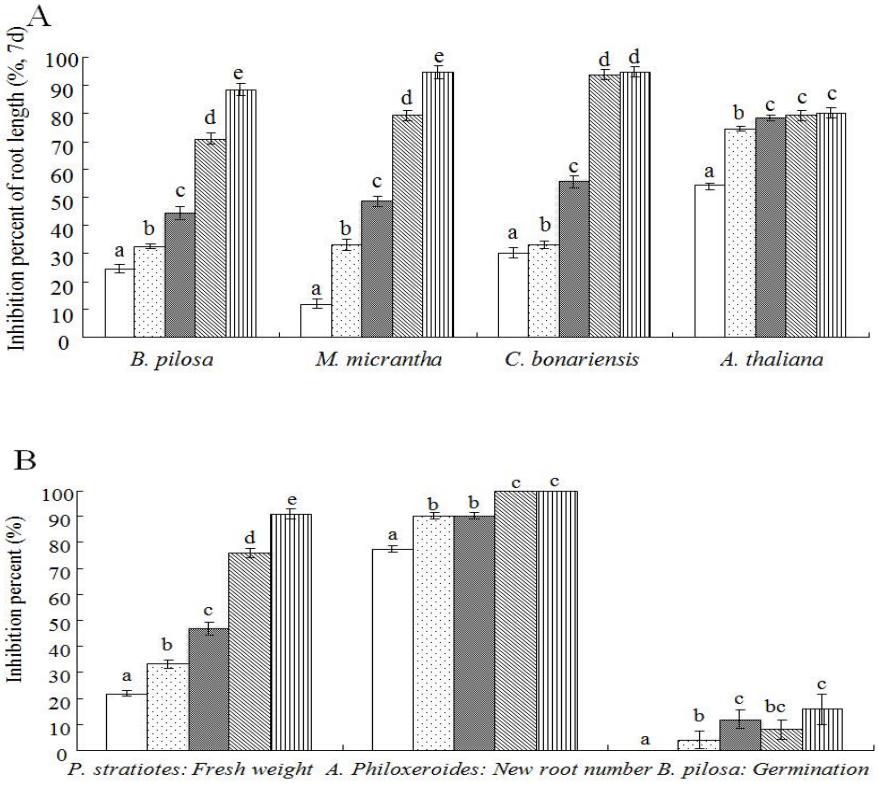


Figure 5. Effects of berberine on *B. pilosa*, *M. micrantha*, *C. bonariensis*, *A. thaliana*, *P. stratic* (with 2-3 leaves) *and A. philoxeroides* .

A: Inhibition of *B. pilosa*, *M. micrantha*, *C. bonariensis* and *A. thaliana* were inhibition rate (% primary root length 7d after berberine treatment. Berberine concentrations used on *B. pilosa* w 1.25, 2.5, 5, 10 and 20 μg/mL (left to right); on *M. micrantha:* at 5, 10, 20, 40 and 80 μg/r on *C. bonariensis at* 3.125, 6.25, 12.5, 25 and 50 μg/mL; *on A. thaliana*: 0.2, 0.5, 1, 1.5 an μg/mL.

B: Berberine inhibited the fresh weight in *P. stratiotes* (with 2-3 leaves) after 15 d. Inhibitior new root number for *A. philoxeroides* was determined after 15 d; Inhibition in germination o *pilosa* was determined after 3 d. Berberine concentrations used on *P. stratiotes* were: 3.125, 6. 12.5, 25 and 50 μg/mL (left to right); *A. philoxeroides*: 3.125, 6.25, 12.5, 25 and 50 μg/mL; *pilosa*: 6.25, 12.5, 25, 50 and 100 μg/mL

Berberine at 12.5 μg/mL reduced the fresh weight of *P. stratiotes* (2-3 leaves stage) by 46.87% at 15 d after the treatment; Berberine at 25 μg/mL completely inhibited (100%) new roots in *A. philoxeroides* after 15d. However berberine at 100 μg/mL, inhibited the germination of *B. pilosa* only by 16.00%, which showed that berberine is less harmful to the germination of *B. pilosa* (Fig. 5B).

Bertin (2007) reported m-tyrosine, isolated from the *Festuca rubra* L. ssp. *commutate*, was strongly inhibitory to *A. thaliana* and the IC_{50} values of L- and D- tyrosine on *A. thaliana* were 0.11 μg/mL (0.6 uM, 5 d) and 1.69 μg/mL(9.3 uM, 5 d), respectively(8). When treated with 0.2 μg/mL of berberine, the inhibition of root length was 54.03% (7d), with the IC_{50} value of berberine on *A. thaliana* was about 0.2 μg/mL suggesting that berbeine herbicidal activity could match with m-tyrosine.

IV. Berberine and herbicides bioassay

At 7 days after the treatment, the IC_{50} values of berberine, glyphosate and pendimethalin on the fresh weight of *B. pilosa* were 7.28, 9.48 and 15.13 μg/mL, respectively (Table 1, Fig. 6) as compared with synthetic herbicides. This indicated that the herbicidal effect of berberine on *B. pilosa* was comparable and even better than glyphosate and pendimethalin herbicides.

V. Berberine bioassay of maize

At 7 days after the treatment, the IC_{50} values of berberine on the fresh weight of maize was 27.40 μg/mL (Table 1). This indicated that berberine is safe to maize crop at 7.28 μg/mL dose to control *B. pilosa* weed.

Table 1. IC_{50} values of berberine on fresh weight of *Zea mays* and IC_{50} values of herbicides (pendimethalin, glyphosate) and berberine on fresh weight of *B. pilosa*

Test plant	Herbicide	Regression equation	IC_{50}(μg/mL)	95% confidence limit (μg/mL)	Correlation coefficient (r)
Maize	Berberine	y =1.23+2.62 *x*	27.40	21.72~34.56	0.97
B. pilosa	Pendimethalin	y=3.69+1.11x	15.13	8.92~25.68	0.99
	Glyphosate	y=3.87+1.16x	9.48	6.20~14.50	0.98
	Berberine	y=4.23+0.89x	7.28	4.26~12.43	0.97

Fresh weights were measured 7 days after sowing.

VI. Mechanism of berberine on *A. thaliana*

The mechanism of berberine as drug in mouse cell lines or human cell lines have revealed its interaction with some receptors, enzymes etc. (1,2,10,16,20,22,30). However, the herbicidal mechanism of berberine is not known. CYCB1(Cyclin b1) is important plant growth regulator to modulate the cell cycle (26,27). As shown above, berberine inhibited the root elongation and root growth. This suggested that berberine might affect the cell division. *A. thaliana* (CYCB1:GUS) was used to test the hypothesis and the Results (Fig. 7) showed that CYCB1 activity in roots treated was affected by berberine. Ten d after the

treatment, no expression of CYCB1 was observed in tips of primary roots when treated with 5 μg/mL berberine, suggesting that berberine completely inhibited the expression of CYCB1 in primary roots. However, the exact herbicidal mechanism of berberine needs further studies.

Figure 6. Effects of berberine (A), glyphosate (B) and pendimethalin (C) on *B. pilosa*, 7 d after the treatment (B-2μg/mL, means "treated with 2μg/mL berberine").

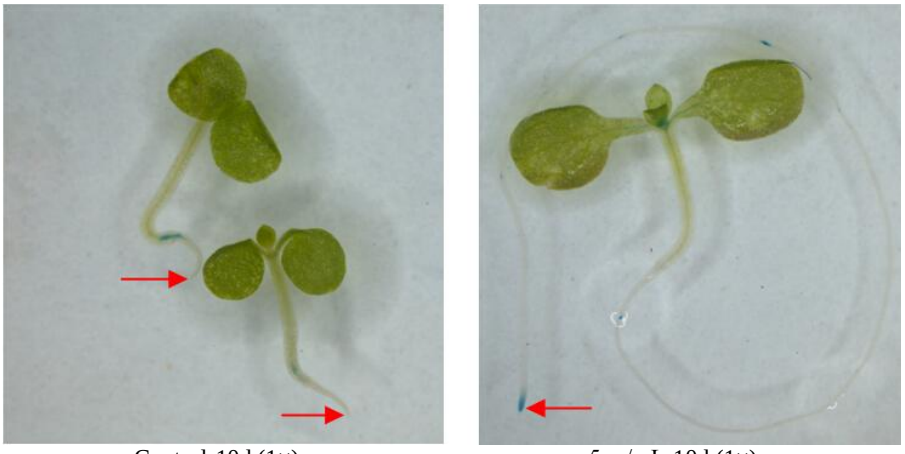
VII. Pot experiment

To find if berberine could be used for weeds control as soil applied herbicide, the pot experiments were done. The berberine at 50-200 μg/g inhibited the fresh weight of root and stem of *B. pilosa* (Table 2). At 50 μg/g, the inhibition in fresh root and stem weight of *B. pilosa* was 42.80% and 61.87%, respectively (Table 2). These results revealed that berberine could be developed as new herbicide.

Table 2. Effects of berberine on *B. pilosa* in pot experiment

Concentration(μg/g)	Inhibition (%) of fresh root weight	Inhibition (%) of fresh stem weight
50	42.80±3.72a	61.87±3.98a
100	65.29±4.20b	68.15±4.35b
200	66.28±5.43b	85.63±5.84c

Growth was measured 60 d after sowing .Values are percent of control
All data represent means ± SD. Values followed by different letters in the same column indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.



Control-10d (1×) 5μg/mL-10d (1×)

Figure 7. CYCB1:GUS activity of *A. thaliana* 10 d after berberine treatment. Blue color in root meristem in control means expression of Cyclin b1, as indicated by the red arrow. No expression of Cyclin b1 in root meristem in the treatment of 5 μg/mL berberine, as indicated as no blue color in root meristem by the red arrow.

CONCLUSIONS

The naturally occurring plant chemical berberine, isolated from *C. chinensis*, significantly inhibited the root growth of 5-invasive weeds (*B. pilosa*, *M. micrantha*, *C. bonariensis*, *P. stratiotes* and *A. philoxeroides*). This study also revealed the mechanism of plant growth inhibition of berberine. It inhibits the cell division in roots of test plant *A thaliana*. The effective dose of berberine on the fresh weight of *B. pilosa* weed was 7.28μg/mL (7d after the treatment), which was significantly lower than the dose to affect the maize growth (27.40 μg/mL). Thus berberine is safe to maize at the effective dose to control the *B. pilosa* weed. Hence, berberine could be developed as new herbicide for weed control.

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