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Table: 1, Figs : 5

Biofertilization alleviates problems of continuous cowpea cropping by degrading autotoxins in soil

X. X. HUANG*, C. F. LIN, B. C. WANG, Z. H. HU, G. L. ZHOU,
A. H. WANG and C. L. YU

Wuhan Institute of Vegetable Science, Wuhan, 430345, China.
E. Mail: xingxuehuang@yahoo.com

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ABSTRACT

The effects of biofertilization on alleviating the problems in continuous cowpea cropping has been examined. Compared with NPK fertilization, biofertilization significantly improved shoot length, total biomass, volume, activity, H^+-ATPase activities, levels of free Spm (spermine) and free Spd (spermidine) in the roots of the continuously cropped cowpea. The addition of exogenous cinnamic acid increased the levels of the cinnamic acid in rhizosphere soils and decreased total biomass, volume, activity and H^+-ATPase level of the root, while biofertilization alleviated these adverse effects. These results suggest that biofertilization helps in alleviating the adverse effects of continuous cowpea cropping by eliminating the autotoxins.

Keywords: Continuous cropping, cowpea, Biofertilization, ATPase,

INTRODUCTION

Cowpea is a crop of major economic importance throughout the world (13). It is generally cultivated in a continuous cropping system and this causes a significant decrease of output and quality (35).This is believed to be due to the soil sickness and autotoxicity, resulting from the accumulation of autotoxins in the soil (21,45,47). Autotoxicity is a process in which a plant or its decomposing residues release toxic chemicals into the environment to inhibit the germination and growth of the same plant (15). Autotoxins enter the soil through plant roots, residue decomposition and foliar leaching by rainwater (19, 24). They act on the target sites of plant cells and influence the membrane permeability, cell differentiation, phytohormone generation, ion uptake, protein synthesis etc., which eventually inhibit plant growth (19). The effects of autotoxins are concentration independent (44). At high concentration, they inhibit growth but promote the growth at lower concentrations, by alleviating the problems caused by autotoxicity (18, 28).

Many measures such as soil disinfection (38), grafting (22), and crop rotation have been examined to alleviate the problem of continuous cropping (33,41). However, all these measures have their limitations (31). One measure used to overcome soil sickness due to autotoxins is by the use of biofertilizers, which have been reported to control soil sickness (9,44), improve the soil nutrient status and the physical environment (1,25,27,46). However there are limited studies on the alleviation of autotoxicity in continuously

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[*] Correspondence author

cropped systems. The present studies aimed to determine the effects of biofertilizer on alleviating continuous cropping obstacles in cowpea cultivation by investigating its effects on growth, plasma membrane H^+-ATPase and vacuolar H^+-ATPase in cowpea roots and the chemical composition of rhizosphere soilss.

MATERIALS AND METHODS

This field study was done at Wuhu institute of vegetable science (30°34'N, 114°16'E), Wuhan, China. Cowpea cultivar (*Vigna unguiculata* (L.) Walp.) "E Cowpea Number 9" was used in this study. The field soil was sandy loam [NH_4^+-N: 122 mg·Kg^{-1}, P: 62.5 mg·kg^{-1}, K: 87.9 mg·kg^{-1}, organic matter: 15.2 mg·kg^{-1} and pH was 5.8}. This field was used to grow cowpea continuously for 8- years. The field was ploughed thoroughly and plots of 26 m^2 (20 m × 1.3 m) were prepared. The cowpea was sown on August 2, and harvested on September 30, 2013. The treatments were replicated thrice in randomized block design. The field experiment consisted of four treatments as under:

Treatment	Details
F	**F [Control (only NPK fertilizer at 1 t/ha)]:** The control plots received only 1 t ha^{-1} NPK fertilizer (N: P: K=15%:15%:15%). The 80% NPK fertilizer was applied as basic manure, applied 3 days before sowing.
BF	**BF (Biofertilizer only at 22 t/ha):** This BF compost was prepared with fresh cow manure, corn straw and 80 microorganisms group. A Pit (1 m deep, 1.5 m wide and 2 m long) was dug on elevated surface to prevent rain water. It was filled with mixture of corn straw + cow manure (2: 1 ratio) layer by layer (each layer 10 cm high) followed by a thin layer of effective microorganisms (CM) obtained from Shanxi Biological Company Limited.. The bacterial group contained more than 80 kinds of effective microorganisms. The top of pit was covered with mud and temporary shade to prevent excess rain water entry. To hasten the decomposition rate, the compost materials were turned over thice every 3-day. The fertilizer was ready for use in 14 days after composting. It contained (1.2% N, 2.1% P, 6.1% K, 24.1% organic matter, 23.1% water, C: N=35.2:1, pH 8.2) and was applied to soil as basic fertilizer 3 days before sowing. No chemical fertilizer was added to these plots.
F+CA	**Ferilizer at 1 T/ha+ Cinnamic acid at 448 kg/ha :** Both 100 g NPK fertilizer and 44.8g cinnamic acid were added per plot (1 m^2) into soil 3 days before sowing during the soil preparation.
BF+CA	**Biofertilizer** at 22 t/ha **+Cinnamic acid at 448 kg/ha:** Both 2.20 kg organic fertilizer and 44.8 g cinnamic acid were added per plot (1 m^2) into soil 3 days before sowing during the soil preparation. No chemical fertilizer was added to these plots.

Plant growth analysis

At 30 days after sowing, 3- plants were randomly selected from each plot. These plants were dug out from the root level by Core Sampler (0.3 m × 0.3 m × 0.3 m soil) and the roots were soaked in tap water for 30 min to remove soil and organic matter. Then roots were dried with paper. The root volume was measured by water displacement method (42), and the root dehydrogenase activity was evaluated by triphenyl tetrazolium chloride (TTC) method (43). The plants shoot length, root biomass, root activity, volume, plasma membrane H^+-ATPase (PM-H^+-ATPase), vacuolar H^+-ATPase (V-H^+-ATPase) activities and contents of polyamines in roots were estimated. The destructive sampling method was used to estimate shoot length and biomass. The biomass was oven-dried at 70 ºC for 48 h to determine dry matter (12).

Soil sample collection

To determine the field soil chemical composition, soil samples of 1 kg per plot were collected on September 23, 2013. The rhizosphere soil (about 5 mm from the root surface) was scraped carefully, collected and air-dried (26). The soils were sieved through 2 mm mesh, and stored (4 °C) in a brown bottle for the chemical analysis of soil.

Soil chemical composition

Rhizosphere soils were extracted using the method of Heimler (14). The soil was air-dried at root temperature (20 °C-25 °C, 25 days). Ten g air-dried soil was stirred in 50 mL distilled water for 24 h. After filtering theough the filter paper and centrifugation (3000 *g*), its 5 mL solution was acidified with 2 mL of 2 M HCl and extracted with 5 mL of ethyl acetate. Three mL of organic phase was concentrated to dryness on a rotary evaporator (34 °C) and completely dried over P_2O_5 under vacuum for 24 h. The dried residue was converted to trimethylsilyl derivatives by adding 0.5 mL of Regisil (99% N,O bis[trimethylsilyl] trifluoroacetamide + 1% trimethylchlorosilane) and 1.16 mL of pyridine and heated at 70°C for 30 min.

GCMS Analysis

After centrifugation, the supernatant solution was used for GC-MS analysis, done within 6 h of silylation process. To determine the compositon of the above extracts for various chemicals, the extracts were analysed using GC-MS as per the method of Seal et al. (30). The GC (Trace)/MS (Polarisq) contained a fused silica capillary column (HP-5, 50 m × 0.25 mm I.D. × 0.33 µm). Helium was used as the carrier gas with at a flow rate of 30 mL min-1 and the injection volume of 1 µL. The temperature gradient was from 80°C to 200°C at 5°C min-1 and the bombarding voltage was 70 eV. The scanning range was 30-650 amu (M/Z) and the scanning took 0.2 sec for the whole sweep.

HPLC Analysis of autotoxic compounds

Ten g of air-dried soil was stirred in 50 mL 95% alcohol for 6 h. After filtertion and centrifugation (3000 *g*), 5 mL solution was extracted with 5 mL ethyl acetate. One mL of organic phase was used for HPLC analysis of autotoxic cmpounds. The concentration of autotoxic compounds in soil samples was determined using the Waters HPLC system (Waters, Milford, MA), s equipped with a Waters 600 pump, a Waters 600 system controller, a 2996 UV-DAD detector, and a model 7752 injector with 30 µL sample loop. All separations were done with an Agilent ODS-C18 column (150 cm × 4.6 mm I.D., 5 µm), with a flow rate kept constant at 0.8 mL min^{-1}. HPLC separations were conducted using the following mobile phase solutions: mobile phase A: acetonitrile, mobile phase B: 1% glacial acetic acid (regulated to pH 2.5 with water). The linear gradient system ranged from 5% to 35% phases A (v/v) over a period of 40 min. The detection was performed at 280 nm. The injection volume was 30 µL and the column temperature was maintained at 25°C. The chromatographic data were recorded and processed using a Prostar work station. The concentrations of compounds in the soil samples were determined based on peak areas using external standards. Four organic compound standards (cinnamic acid, phenylacetic acid, 4-hydroxybenzoic acid and phthalic acid) used were from Sigma.

ATPase and polyamine content: The activities of ATPase in the root were measured as the release of inorganic phosphate from hydrolysis of ATP as per the methods of Ayala1 (3) and expressed as μmol Pi·mg^{-1} protein·h^{-1}. The free polyamine levels in the root were measured as per the method of Huang and Bie (17) and expressed as nmol·g^{-1}DW.

Statistical analysis: Statistical analysis was carried out by SAS 8.1 to evaluate significant differences between means at $p<0.05$. Excel 2003 was used for correlation analysis and graphical presentations.

RESULTS

Shoot length and biomass: Compared with the control (NPK fertilizer treatment only) plants, the plants in plots treated with biofertilizer significantly increased the shoot length and biomass by 86.7% and 102.7%, respectively. CA treatment decreased the shoot length and the biomass by 32.9% and 43.4%, respectively as compared with the control. However, the application of biofertilizer significantly alleviated the changes induced by cinnamic acid alone treatment (Figure 1).

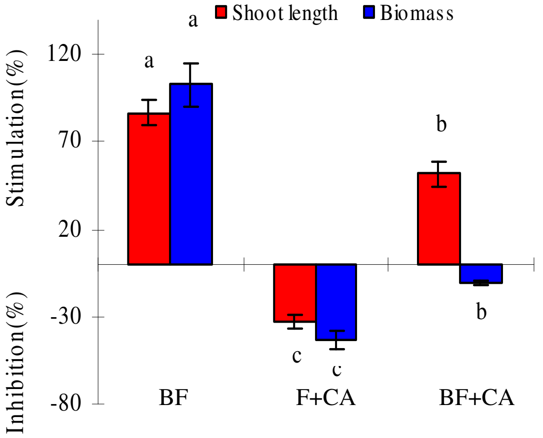

Figure 1. Effects of BF (Bio fertilizer), F+CA (NPK fertilizer+ Cinnamic acid), BF+C A (Bioferilizer + Cinnamic acid) on the shoot length and biomass of continuously cropped cowpea. Each value is the mean ± standard error of the mean (SE) of triplicates. Values with the same letter are not significantly different at $P<0.05$.

Root volume and root activity: Compared with the control, the cowpea plants treated with biofertilizer showed significantly increased root volume and root activity by 71.8% and 31.0%, respectively. Addition of cinnamic acid decreased the root volume and the root activity by 62.9% and 62.1%, respectively, while the application of the biofertilizer significantly alleviated the changes induced by only cinnamic acid treatment (Figure 2).

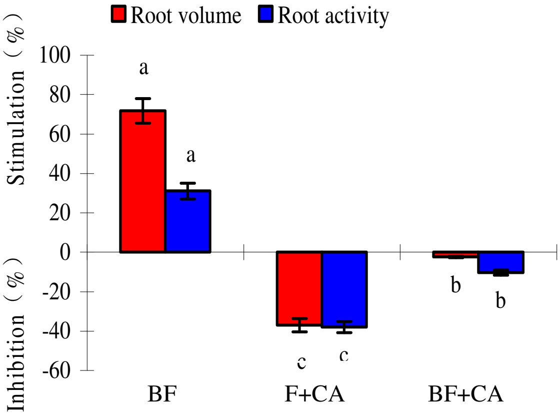

Figure 2. Effects of BF (Bio fertilizer), F+CA (NPK fertilizer+ Cinnamic acid), BF+C A (Bioferilizer + Cinnamic acid) on the root volume and root activty of continuously cropped cowpea. Each value is the mean ± standard error of the mean (SE) of triplicates. Values with the same letter are not significantly different at $P < 0.05$.

V-H+-ATPase and PM-H+-ATPase activities: Compared with the control, the activities of V-H+-ATPase and PM-H+-ATPase in the roots of plants from plots treated with biofertilizer were also significantly higher while the plants treated with cinnamic acid showed decreased activities of these enzymes. The adverse effects induced by only cinnamic acid treatment were significantly alleviated by the application of biofertilizer (Figure 3).

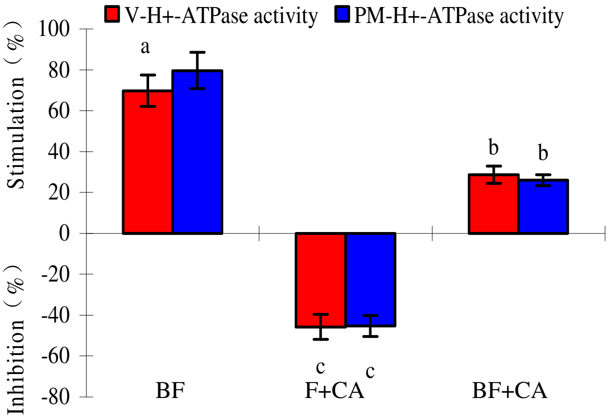

Figure 3. Effects of BF (Bio fertilizer), F+CA (NPK fertilizer+ Cinnamic acid), BF+C A (Bioferilizer + Cinnamic acid) on the V-H$^+$-ATPase activities and PM-H$^+$-ATPase activities of continuously cropped cowpea. Each value is the mean ± standard error of the mean (SE) of triplicates. Values with the same letter are not significantly different at $P < 0.05$.

Soil Chemical composition

Comparing the GC-MS results of rhizosphere soils with the GC-MS user-library spectra of pure reference compounds, 46 and 40 principal chromatographic peaks were detected in extracts of rhizosphere soils of continuously cropped cowpea treated by biofertilizer and NPK fertilizer, respectively (Table 1). Thirty eight compounds were found in the two rhizosphere soil extracts: Control and BF raetments, but their relative contents differed. Soil treated with BF (biofertilizer) contained: 7 alkanes (7.5%), 10 esters (18.17%), 4 fatty acids (24.87%), 3 ketones (8.19%), 2 aldehydes (2.12%), and 8 phenols (4.59%). Soil treated with NPK fertilizer contained: 8 alkanes (14.26%), 7 esters (11.54%), 3 fatty acids (5.23%), 2 ketones (2.02%), 2 aldehydes (3.63%) and 9 phenols (15.19%). The unique compounds in the extract of soil treated with biofertilizer were alanine, crotonyl alcohol, propanoic acid, propanediol, allyl benzene, and diethyl succinate, while the unique compounds in the extracts of soil treated with NPK fertilizer were o,p'-DDE and 1,10-cycloeicosanedione (Table 1). The biofertilizer changed the chemical composition of soil (Table 1) as evident from the compounds present in the rhizosphere.

Phenolic acids content: Compared with the control, BF treatment significantly decreased the concentrations of cinnamic acid, phenylacetic acid, 4-hydroxybenzoic acid and phthalic acid of the soils by 92.5%, 81.0%, 87.2% and 88.3%, respectively. However, cinnamic acid treatment only increased the concentration of cinnamic acid and not of other three phenolic acids. The BF +CA treatment significantly decreased the concentrations of cinnamic acid, phenylacetic acid, 4-Hydroxybenzoic acid and phthalic acid in soils under continuous cowpea cropping by 80.7%, 81.8%, 88.5 and 88.8%, respectively, than CA treatment (Figure 4).

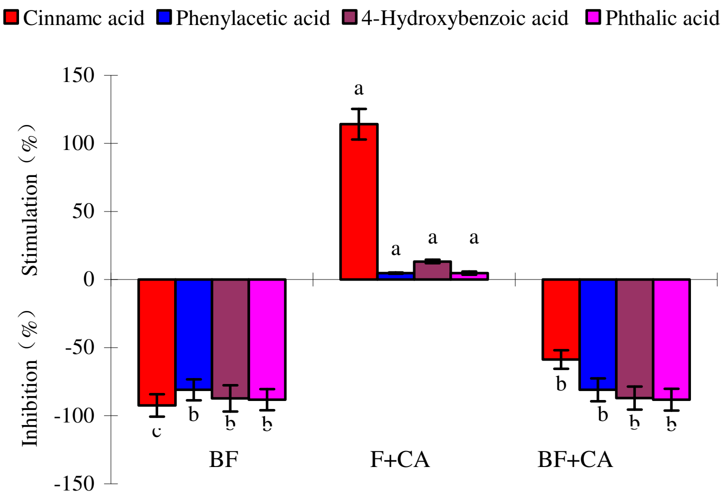

Figure 4. Effects of BF (Bio fertilizer), F+CA (NPK fertilizer+ Cinnamic acid), BF+C A (Bioferilizer + Cinnamic acid) on the concentrations of the four phenolic acids in soils under of continuous cowpea cropping. Each value is the mean ± standard error of the mean (SE) of triplicates. Values with the same letter are not significantly different at P <0.05.

Table 1. Chemicals in rhizosphere soils extracts of continuously cropped cowpea with different fertilizers

Peak	Retention time (min)	Compounds	Peak area of treatments (%)	
			Biofertilizer	Control (NPK fertilizer)
1	4.78	Alcohol	0.81	0.32
2	5.24	Alanine	0.54	—
3	5.67	Butyl ether	2.12	1.07
4	6.63	Acetic acid	2.63	0.51
5	6.96	Butanone	0.42	0.12
6	7.49	Acetic ether	2.52	1.87
7	8.43	Crotonyl alcoho	0.62	—
8	9.01	Propanoic acid	1.52	—
8	9.42	Butyraldehyde	0.87	0.52
9	9.63	Ethyl propionate	1.65	0.13
10	9.75	Phthalic acid	0.42	1.82
11	9.95	2,6-Bis(1,1-dimethylethyl)-2,5-cyclohexadiene-1,4-dione	0.33	0.67
12	10.36	Phenol, 2,5-bis(1,1-dimethylethyl)-	0.43	1.62
13	10.44	1,54-dibromo-Tetrapentacontane	0.23	0.65
14	10.93	Cinnamic acid	0.51	3.75
15	11.61	Isobutyric acid	1.12	2.22
16	11.82	Propanediol	1.31	—
17	12.29	4-Hydroxybenzoic acid	0.34	1.54
18	12.62	Caproic aldehyde	4.24	3.11
19	12.96	12-Methyltridecanoic acid methyl ester	2.59	2.03
20	13.18	9-Hexylheptadecane	0.54	1.24
21	13.43	2,6-Bis(1,1-dimethylethyl)-4-ethylphenol	2.03	3.19
22	13.52	Phenylacetic acid	0.32	0.85
23	13.82	12-Methyltetradecanoic acid methyl ester	0.66	0.68
24	14.21	Pentadecanoic acid methyl ester	0.86	0.52
25	14.47	6,10,14-Trimethyl-2-pentadecanone	1.35	1.23
26	14.81	E-8-Methyl-9-tetradecen-1-ol acetate	1.5	1.97
27	14.93	9-Octyl-heptadecane	0.81	0.79
28	15.21	Tetratetracontane	1.38	1.35
29	15.40	Trans-2-hexadecenoic acid	3.85	6.35
30	17.04	7-Hexyleicosane	1.67	2.61
31	18.23	17-Pentatriacontene	0.54	1.03
32	18.59	7, 10-Octadecadienoic acid methyl ester	3.14	3.34
33	18.71	(E)-9-Octadecenoic acid methyl ester	2.98	2.99
34	19.31	2, 3-Dihydroxypropyl elaidate	3.82	3.08
35	20.05	o,p'-DDE	—	1.12
36	20.43	9-Hexylheptadecane	1.64	3.13
37	20.55	1,10-Cycloeicosanedione	—	1.4
38	22.53	Eicosanoic acid methyl ester	1.33	0.93
39	23.72	Allyl benzene	0.21	—
40	24.27	Heptenyl methanal	1.32	0.68
41	27.86	Diethyl succinate	1.09	—
42	28.87	11-Decyltetracosane	1.23	3.09
43	29.47	Ethyl caprylate	1.35	0.25
44	29.76	2,4-Heptadiene	0.25	1.02
45	30.06	Phenylethyl acetate	0.37	0.63
46	31.42	Hendecanoic acid	0.22	1.64

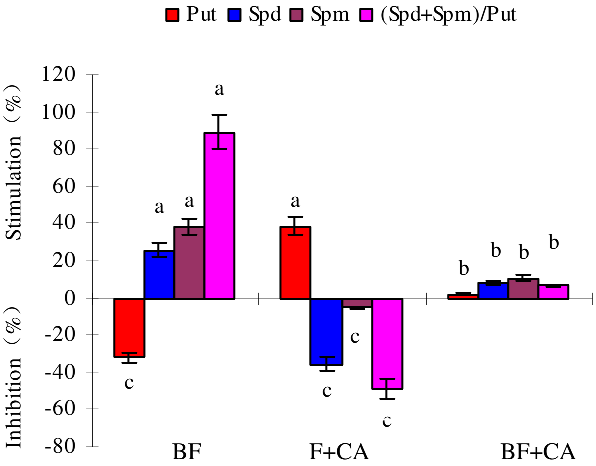

Figure 5. Effects of BF (Bio fertilizer), F+CA (NPK fertilizer+ Cinnamic acid), BF+C A (Bioferilizer + Cinnamic acid) on the levels of the free polyamines (PAs) in the root of continuously cropped cowpea. Each value is the mean ± standard error of the mean (SE) of triplicates. Values with the same letter are not significantly different at P <0.05.

Polyamines content: Compared with the control treatment, the biofertilization treatment significantly decreased the level of free polyamines by 32.0%, but significantly increased the levels of free Spd, (Spermidine) free Spm (Spermine) and total free Polyamines by 25.8%, 38.6% and 18.3%, respectively. The CA treatment significantly increased the level of free Put (Putrescine) by 38.5%, but significantly decreased the levels of free Spd, free Spm and total free PAs by 35.5%, 35.6% and 18.3%, respectively (Figure 5).

DISCUSSION

Plant Autotoxins are major obstacles in continuous cropping, which lead to replant disease (6). Many measures have been taken, such as crop rotation, grafting, soil sterilization, balanced fertilization to relieve the replanting problem. However, these measures are ineffective since rotation needs enough time and space; grafting can only be used for a few varieties of crops; fertilization can not solve the problems of soil sickness and autotoxicity (15). On the other hand, organic fertilizers are known to improve the biological quality (5,37) as well as alleviate autotoxicity (32). In the present study, we have examined the beneficial effect of biofertilization to alleviate the continuous cropping obstacles. The biofertilizer significantly increased biomass, plant height and root volume of cowpea (Fig. 1 and Fig. 2), which corroborates the results of Wang and Wang (36). The application of effective microorganisms group and organic matter may have been responsible for this beneficial effect. This is suggested by the chemical compositon of the rhisosphere soil extracts for various phenolics.

The comparison of the rhisophere soil chemical compositions showed that the use of biofertilizer decreased the levels of the four phenolic acids (Table 1, Figure 4), which are known to be autotoxins in continuous cowpea cropping (16), The levels of the autotoxins in the continuously cropped soils were closely related to the biomass and root volume, which is similar to the results of Zhang *et al.* and Du *et al.* (11,40). The decreased levels of the autotoxins may be attributed to the degradation by the microorganisms in soils (2,10) and /or the absorption by organic matter (8). The increased root volume also decreased the levels of autotoxins which comes in contact with per unit root surface, which is more closely related to the factual autotoxicity effects than the total content of autotoxins in the soil (23).

Root is the primary site of attack by the autotoxins. Phenolic acids depolarize the root membrane potential, and subsequently the functions of the membrane are disturbed, such as inactivation of membrane-associated enzymes, ion leakage etc, which finally decreases the absorption and transportation capability of the roots (4, 20). The growth of the cowpea in continuously cropped area was improved because both the root volume and root activities were enhanced by the biofertilizer (Fig. 3). The increasing of H^+-ATPase activity indicated an improvement of the root membrane, which may be partly attributed to the elevated levels of root Spd and Spm that protect the enzyme (34). Spd and Spm serve as a homeostatic buffering system to stabilize the intracellular pH and maintain a cation–anion balance in plant tissues (29). Free Spd and free Spm might interact with the membranes by inhibiting the trans-bilayer movement of phospholipids (7). It was also testified by the results of the polyamine determination (Fig. 5).

CONCLUSIONS

The biofertilization improved the growth and root functions of continuously cropped cowpea by decreasing the concentration of autotoxins in rhizosphere soil. These results suggest that biofertilizer may help to alleviate the problems of continuous cowpea cropping by degrading the phenolic acids and by increasing the ratio of polyamines.

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