https://doi.org/10.26651/allelo.j/2018-43-1-1135 0971-4693/94 Euro 20.00
*Allelopathy Journal* 43 (1): 117-130 (January, 2018) *International Allelopathy Foundation* 2018
Table: 5, Figs : 6

# Effects of vanillic acid on peanut seed germination, seedling growth and rhizosphere microflora

Y.Q. Huang, X.R. Han, C.H. Liang[1], C.Q. Zang[1], R.X. Shao,
Y.Q. Zeng and J.F. Yang*
Land and Environment College, Shenyang Agricultural University,
No. 120 Dongling Road, Shen He District, Shenyang City,
Liaoning Province, 110866. China
E. Mail: hyqlch@163.com

**(Received in revised form: November 03, 2017)**

## ABSTRACT

The toxicity of vanillic acid on seed germination and seedling growth of peanut and its effects on rhizosphere microflora was investigated. The results showed that seed germination and other plant growth characteristics were significantly affected by vanillic acid at concentration > 0.01 mM.L$^{-1}$. The number of rhizosphere bacteria and actinomycetes were markedly reduced with an increase in vanillic acid concentration. Higher concentration also inhibited the rhizosphere fungi but lower concentrations were stimulatory.

**Keywords:** *Arachis hypogaea*, autotoxic substances, microflora, peanut, photosynthesis, rhizosphere, root activity, seed germination, seedling growth, vanillic acid.

## INTRODUCTION

Peanut (*Arachis hypogaea* L.) is major oil seed crop in China (25) and grown continuously in monoculture due to land scarcity and agro-industrialization. Continuous cropping (CC) has however, caused severe yield losses; greater the number of successive planting years, the greater is the loss in yield (31,45,47). The mechanisms of CC on yield reduction are complex and not well understood. Many factors (Autotoxicity, soil physico-chemical properties and degradation of soil environment) are suggested as factors contributing to crop losses. Of these, autotoxicity (a type of allelopathy) is major factor. Autotoxicity has been reported in many crops [Rice, wheat, barely, corn, cucumber, asparagus, apple, tea etc. (4,6,15,16,36,38,39,40)]. Autotoxic plants release toxins that adversely affects the ion uptake, membrane permeability, photosynthesis and phytohormone balance and thereby affects the growth of the same plants in subsequent plantings. Plants release autotoxins through root exudates, leachates volatiles and plant residues. Many of these plant chemicals (terpenoids. steroids, phenols, coumarins, flavonoids, tannins, alkaloids, and cyanogenic glycosides etc.) are known to produce toxic effects (30). Among these, phenolic acids have been intensively studied for their phytotoxicity and have been suggested to cause autotoxicty problems in continuous cropping systems (8,28).

*Correspondence author, [1]Plant Protection Research Institute, Liaoning Academy of Agri.l Sciences, No. 84, Dongling Road, Shen He District, Shenyang City, Liaoning Province, 110161. China.

Our previous studies (13) have shown that the aqueous extracts of peanut plants are inhibitory to its own seed germination and seedling growth and the degree of inhibition increases with increasing extract concentration. Four phenolic acids (p-hydroxybenzoic acid vanillic acid, coumaric acid and coumarin) were detected and quantified by HPLC in the peanut rhizospheric soil extract. Of these, vanillic acid and coumarin were in higher concentrations than other phenolic acids and their concentration in Rhizospheric soil increased with the number of years of continuous cropping (13). However no systematic studies have been done to assess the toxic effects of these phenolic acids on peanut plants or their effect on soil microflora. This study aimed to evaluate the toxic effects of vanillic acid on seed germination and seedling growth of peanut and also on the rhizosphere microorganisms during the growth of peanut plants.

## MATERIALS AND METHODS

Healthy seeds of peanut variety 'Fuhua 12' were used in this study. Vanillic acid (VA) was purchased from Acros Organics (http://www.acros.com/). Six-concentrations of vanillic acid (0,0.01,0.03,0.05,0.07,0.09 $mM.L^{-1}$) were prepared (Table 1). Peanut seeds were surface-sterilized with 0.5% sodium hypochlorite solution for 2 min, then washed five times with distilled water and dried between two paper towels. Six seeds were sown per Petri dish (9 cm dia) lined with Whatman No. 1 filter paper and 2 ml vanillic acid solution or distilled water (as control) were added as per treatments. The treatments were replicated thrice in random complete block design. All Petri dishes were covered and kept in dark in an incubator at 25 ± 1°C. The number of seeds germinated were counted daily and the seeds were considered germinated, when the emergent radical reached half the seed length. During the experimental period, 1-2 mL distilled water was added daily per Petri dish. After 6 days, the germination potential (% GP), and after 8 days the final germination % (% FGP), germination index (GI) and root length (RL cm), were calculated as under:

$$FGP = n / N \times 100$$
$$G\,P = m / N \times 100$$
$$G\,I = \sum(Gt/Dt)$$

Where, N: Total number of seeds per replication, n: Number of germinated seeds after 8 days, m: Number of germinated seeds after 6 days, Dt: Number of days from the beginning of experiment and Gt: Number of germinated seeds on day Dt.

**Cup culture**

Sand + Perlite mixture was used to examine the toxicity of VA (Vannilic acid) to peanut seedlings. For this, 300 g sterilized sand (High-pressure steam sterilization, 121℃, 60 min) was mixed with 20 g perlite and the mixture was placed in plastic cups (400 mL) and watered before sowing. There were 5-treatments of VA [(0, 0.01, 0.03, 0.05, 0.07 $M.L^{-1}$ concentration (Table 1)]. Uniformly germinated peanut seeds soaked for 4 h in 40℃ water and sown in the Petri dish lined with Whatman No. 1 filter paper were selected and one germinated seed was planted per cup. Each treatment consisted of 3-replicates and each replicate consisted of 10 seedlings. Randomised complete block design was used. Each cup was initially irrigated with 150 mL Hoagland's nutrients solution (24) and 10-20 mL Hoagland's nutrient solution was added daily to provide water and nutrients. Distilled water was added, when necessary during the growth period. The cups were kept in an air-conditioned room [24 ± 1°C and 14 h light/10 h dark photoperiod, 400 µmol photo.nsm$^{-2}$.s$^{-1}$]. Twenty mL VA solutions of different concentrations were added per cup after 3 days. The effective concentrations of vanillic acid were 0.112, 0.336, 0.56, 0.784 µg.g$^{-1}$ sand + perlite mixture. At the seedling stage (20 days after planting) plant height (H), root length (RL), fresh weight, (FW) dry weight (DW)s and chlorophyll content were determined.

Height and RL was calculated from the base of seed to the top of main stem and the root tip, respectively. Fresh weight was determined by weighing the randomly selected plants (3-plants per replication). DW was determined by drying the randomly selected plants (3-plants per replication) in oven at 70°C for 48 h. Chlorophyll content was determined by chlorophyll meter (CCM-200 plus, USA) from the central part of leaf, avoiding the midribs**.** Thirty leaves were analysed per treatment (randomly selected 3-plants per replication). The chlorophyll control was measured 5-times for each leaf and then the mean value per leaf was calculated (27).

Net photosynthetic rate, stomatal conductance and intercellular $CO_2$ concentration were determined with portable photosynthesis system (LI-6400; LI-COR, Inc.). Fully developed and sun-exposed leaves at similar position were selected to measure these parameters on sunny days. MDA (Malondialdehyde) content was determined with thiobarbituric acid-malondialdehyde (TBA-MDA) assay method (11**)**. Root activity, root total absorption area and active absorption area were determined as per Hao (10).

**Response Index:** The response index (RI) (35) is measure of the toxic effects of VA on peanut seed germination and seedling growth and was calculated as under:

$$RI = 1\text{-}C/T, \text{ when } T \geq C$$

$$RI = T/C\text{-}1, \text{ when } T < C$$

Where, C: Control value and T: Treatment value. When RI >0, it represents a promoting effect and when RI <0, it represents inhibitory effect. The control RI is 0.

**Pot experiment**

To determine the effects of vanillic acid on rhizosphere microorganisms, pot experiments were conducted in 2015 at Peanut Scientific Research Centre, Shenyang Agricultural University, Shenyang, China (123°33′E, 40°48′N, temperate humid and semi-humid monsoon climate, frost-free period: 148 to 180 d, annual rainfall: 547 mm and the mean annual temperature is 7.0-8.1℃). Seeds from healthy, good quality peanut pods L. variety ‘Fuhua 12’ were used. Plants were grown in plastic pots (32 × 25 × 22 cm) in glass greenhouse (27 ± 5°C and 65 ± 10% relative humidity). Each pot contained 10 kg (unsterilized) dry soil. The soil was sandy loam and contained 13.1 g.kg$^{-1}$ organic matter, 0.53 g.kg$^{-1}$ total N, 0.67 g.kg$^{-1}$ total P, 18.8 g.kg$^{-1}$ total K, 56.2 mg.kg$^{-1}$ available N, 12.5 mg.kg$^{-1}$ available P, 89.6 mg.kg$^{-1}$ available K, and pH, 6.8 (Unpublished). The soil was fertilized with 0.05 g.kg$^{-1}$ N, 0.1 g.kg$^{-1}$ $P_2O_5$, 0.1 g.kg$^{-1}$ $K_2O$. Vanillic acid was tested at 5-concentrations (0, 0.05, 0.10, 0.15, 0.20 µg.g$^{-1}$ dry soil) (Table 1). Soil, fertilizer and VA (dissolve with 1 mL alcohol, then add 10 mL water) were mixed before sowing at 0, 0.5, 1.0, 1.5, 2.0 mg/10 kg dry soil. Each treatment consisted of 3-replicates and each replicate consisted of 5 pots. Random complete block design was used. Seeds were sown on May 20, 2015 (four seeds/pot). The peanut seedlings were watered regularly (2 L per pot every four days) with distilled water.

Table 1. Details of treatments in various experiments

| No. | Petri dish experiment | | Nursery pot experiment | | Pot culture experiment | |
|---|---|---|---|---|---|---|
| | Treatment | Concentration ($mmol \cdot L^{-1}$) | Treatment | Concentration ($mmol \cdot L^{-1}$) | Treatment | Amount ($\mu g \cdot g^{-1}$dry soil) |
| 1 | Control | 0 | Control | 0 | Control | 0 |
| 2 | $M_1$ | 0.01 | $Y_1$ | 0.01 | $P_1$ | 0.05 |
| 3 | $M_2$ | 0.03 | $Y_2$ | 0.03 | $P_2$ | 0.10 |
| 4 | $M_3$ | 0.05 | $Y_3$ | 0.05 | $P_3$ | 0.15 |
| 5 | $M_4$ | 0.07 | $Y_4$ | 0.07 | $P_4$ | 0.20 |
| 6 | $M_5$ | 0.09 | - | - | - | - |

Rhizosphere soil samples were collected from each treatment at the seedling stage (10 days after sowing), blossom stage (40 days), pod stage (70 days) and maturity stage (100 days after sowing). Peanut plant roots from each pot were carefully separated from the soil and softly shaken. The soil collected by shaking the roots was termed as bulk soils. Rhizosphere soil was collected from the roots by first removing all visible bulk soil by shaking and then the rhizosphere soil was collected by gentle brushing of root surface (3). Each treatment consisted of 3-replicates and each replicate were randomly selected 1 pods. The soil samples were analysed at each sampling.

Total microbial populations were determined by soil dilution plating on various agar media: Tryptic soy agar 0.1% (TSA) for total bacterial number, Potato dextrose agar (PDA) with 50 mg chlortetracycline added, for total fungal counts and water agar antibiotic medium for actinomycetes (17). Bacterial and actinomycetes plates were incubated at 28℃ for 3 and 10 days, respectively and fungal plates at 25℃ for 7 days.

**Statistical analyses :** All data were subjected to one-way ANOVA and means were compared with least significant difference (LSD) and Duncan's multiple range test at 5% level. All statistical analyses were conducted using SPSS (version 11.5).

## RESULTS

### Seed germination

Vanillic acid significantly inhibited the peanut seed germination, seed germination potential, final germination (%) and germination index (Table 2). With an increase in VA concentration, FGP, GP and GI were all proportionately decreased and the toxic effects were gradually increased (Fig 1). These results show that the inhibitory effects of VA on seed germination were concentration dependent.

Table 2. Effects of vanillic acid on the peanut seed germination indices in petri plate bioassay

| Treatment | Final Germination (%) | Germination Potential (%) | Germination Index | Root Length (cm) |
|---|---|---|---|---|
| Control | 1.00a | 0.83±0.17a | 1.27±0.15a | 3.98±2.12a |
| M1 | 0.78±0.25ab | 0.78±0.38a | 0.96±0.39ab | 3.78±0.38a |
| M2 | 0.94±0.10a | 0.89±0.10a | 1.01±0.10ab | 3.84±0.45a |
| M3 | 0.67b | 0.61±0.10ab | 0.70±0.03bc | 3.46±0.42a |
| M4 | 0.78±0.19ab | 0.61±0.35ab | 0.76±0.24bc | 3.39±1.01a |
| M5 | 0.61±0.10b | 0.28±0.25b | 0.56±0.03c | 2.40±0.35a |

Note: Different letters indicate significant differences among treatments at the 5% level.

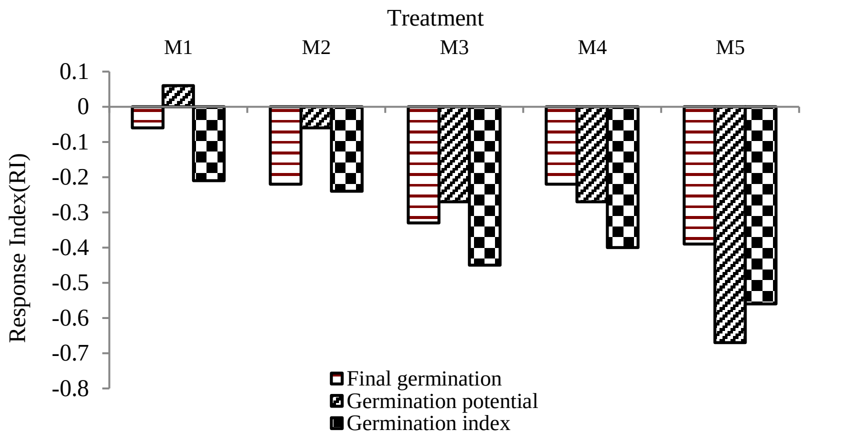


Figure 1. Effects of vanillic acid on Peanut seed germination indices in petri plate bioassay

At VA concentration of 0.09 mM.L$^{-1}$ (M5), the FGP was 61 %, GP was 28 % and GI 0.56, compared with control and were decreased by 39 %, 66.3 % and 55.9 %, respectively. These results also show that the GP was more sensitive to the concentration variation of VA. When VA was 0.01 mM.L$^{-1}$ (M1), GP was 89 % and was 6.0 % higher than control, since its RI was 0.06 (RI> 0), showing a stimulatory effect. At 0.09 mM.L$^{-1}$ (M5), GP declined more than FGP and the toxic effect was greatest. It was found that seed germination was stimulated at low concentrations but inhibited at higher concentrations.

**Seedling growth**

Vanillic acid influenced the growth of peanut seedlings and had harmful effects (Table 3). Different concentrations of VA decreased the height, root length, fresh weight and dry weight of peanut seedlings than in control. This suggested that VA was inhibitory to the seedling growth. Two growth indicators, namely root length and dry weight were decreased by the increase in VA concentration. Vanillic acid at **0.07** mM.L$^{-1}$ concentration (Y4), decreased the seedling root length and dry weight by 37.3 % and 40.0 %, respectively, than control (Fig 2.). Plant height and fresh wt also showed the same trend.

Table 3. Effects of vanillic acid on the peanut seedling growth in Nursery pot experiment

| Treatment | Height (cm) | Root length (cm) | Fresh weight (g) | Dry weight (g) |
|---|---|---|---|---|
| Control | 14.04±1.31a | 8.30±1.50a | 4.24±0.86a | 0.80±0.07a |
| Y1 | 12.56±1.36a | 6.82±1.68ab | 4.04±1.08a | 0.69±0.23ab |
| Y2 | 13.06±0.88a | 5.28±1.21b | 4.20±0.94a | 0.67±0.17ab |
| Y3 | 12.64±1.66a | 5.22±1.19b | 3.80±1.09a | 0.54±0.09b |
| Y4 | 12.28±1.40a | 5.20±0.92b | 3.28±0.36a | 0.48±0.15b |

Note: Different letters indicate significant differences among treatments at the 5% level

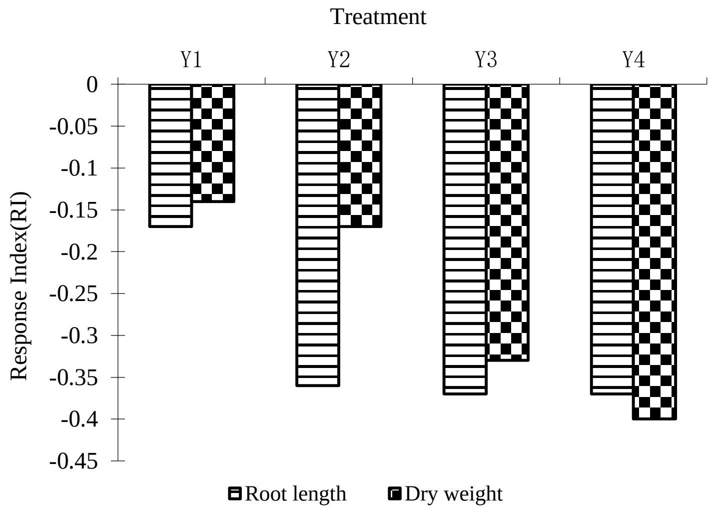


Figure 2. Effect of vanillic acid on the Peanut seedlings growth in Nursery pot experiment

**Photosynthesis**

VA had strong impact on the photosynthetic characteristics of peanut seedlings (Table 4). The chlorophyll content, net photosynthetic rate and stomatal conductance of peanut seedlings treated with different concentrations of VA were all decreased than in control, while the intercellular $CO_2$ concentration was higher than in control. The degree of inhibition increased with the concentration of VA.

**Root activity**

Vanillic acid also influenced the root activity of peanut seedlings (Table 5). The root total absorption area, active absorption area and root activity (active absorption area / total absorption area) of roots treated with VA, were all lower than in control and the effect was concentration dependant. When VA concentration was 0.07 mM.$L^{-1}$ (Y4), the total absorption area, active absorption area and root activity decreased by 22.4 %, 54.2 % and 40.6 %, respectively.

Table 4. Effects of vanillic acid on the peanut plant photosynthetic characteristics in pot culture

| Treatment | Chlorophyll content (%) | Net photosynthetic Rate ($\mu mol \cdot m^{-2}s^{-1}$) | Stomatal conducance ( $mmol \cdot m^{-2}s^{-1}$) | Intercellular $CO_2$ concentration ($\mu mol \cdot mol^{-1}$) |
|---|---|---|---|---|
| Control | 51.63±9.81a | 4.97±0.85a | 93.25±8.94a | 476.51±19.76b |
| Y1 | 45.49±9.42ab | 3.32±0.68ab | 75.11±7.40b | 531.68±10.64ab |
| Y2 | 46.62±7.41ab | 4.36±0.27ab | 68.12±6.57c | 626.50±13.71ab |
| Y3 | 43.01±5.67b | 2.58±0.96b | 58.39±9.70d | 659.08±22.87ab |
| Y4 | 41.81±9.97b | 2.29±0.05b | 47.45±3.99e | 696.19±17.89a |

Note: Different letters indicate significant differences among treatments at the 5% level.

Table 5. Effects of vanillic acid on the peanut root activity in Nursery pot experiment

| Treatment | Total absorption area ( $m^2$) | Active absorption area ( $m^2$) | Root activity |
|---|---|---|---|
| Control | 0.152±0.008a | 0.048±0.006a | 0.32±0.007a |
| Y1 | 0.141±0.007a | 0.040±0.009a | 0.28±0.008a |
| Y2 | 0.127±0.008b | 0.031±0.007b | 0.24±0.007b |
| Y3 | 0.122±0.005c | 0.027±0.003c | 0.21±0.004c |
| Y4 | 0.118±0.003c | 0.022±0.005c | 0.19±0.004c |

Note: Different letters indicate significant differences among treatments at the 5% level.

**MDA content of leaves**

Application of VA also affected the leaf MDA content (Fig. 3) and increased the MDA content of leaves than in control. At 0.03 $mM.L^{-1}$, 0.05 $mM.L^{-1}$and 0.07 $mM.L^{-1}$ (Y2, Y3 and Y4) the MDA content of leaves was significantly increased by 30.9 %, 39.5 % and 43.3 %, respectively, than in control.

**Microflora in peanut rhizosphere soil**

The number of bacteria in the rhizosphere soil varied from the seedling stage to maturity stage in the presence of vanillic acid at various concentrations (Fig. 4). Generally, during the whole growth period, the bacterial numbers in the rhizosphere soil showed an increasing trend but their numbers in all treatments were lower than in control. The reason perhaps is that VA increases the diversity of bacteria in the soil leading to a decline in the number of specific bacteria.

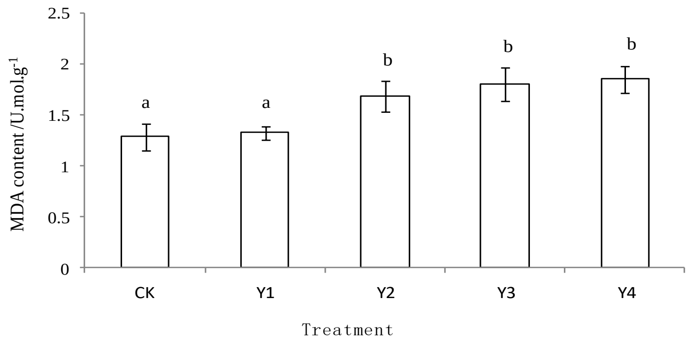


Figure 3. Effect of vanillic acid on the MDA content in Peanut leaves in Nursery pot experiment

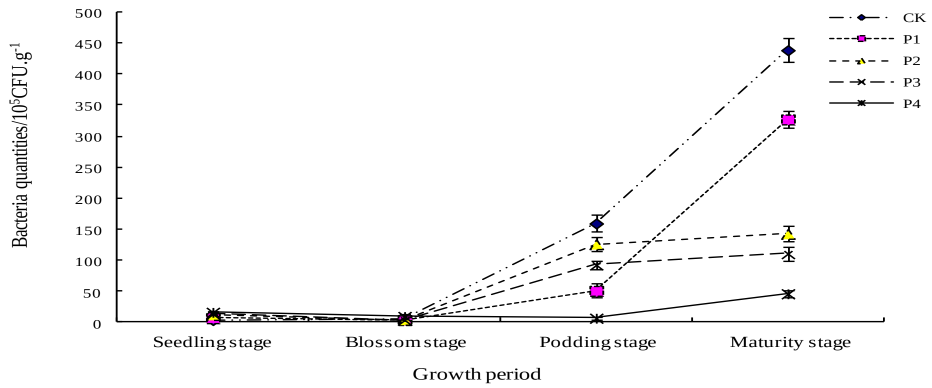


Figure 4. Effects of Vanillic acid on the Number of bacteria in the Rhizosphere soil in pot culture

The number of rhizosphere bacteria among all treatments did not differ significantly from seedling stage to blossom stage. The number in CK and P1 treatment rapidly increased from blossom stage to maturity stage and reached the peak at maturity. Their numbers, however, gradually increased in P2-P4 treatments. This showed that VA had inhibitory effects on the peanut rhizosphere bacteria and the extent of inhibition was concentration dependent.

VA also affected the number of actinomycetes in the peanut rhizosphere (Fig 5) from the seedling stage to maturity. Their number was maximum at the seedling stage and thereafter decreased to a minimum at plant maturity. Like the bacteria, their number in all treatments was lower than in control. These results showed that VA had inhibitory effects on the peanut rhizosphere actinomycetes and the extent of inhibition was concentration dependent.

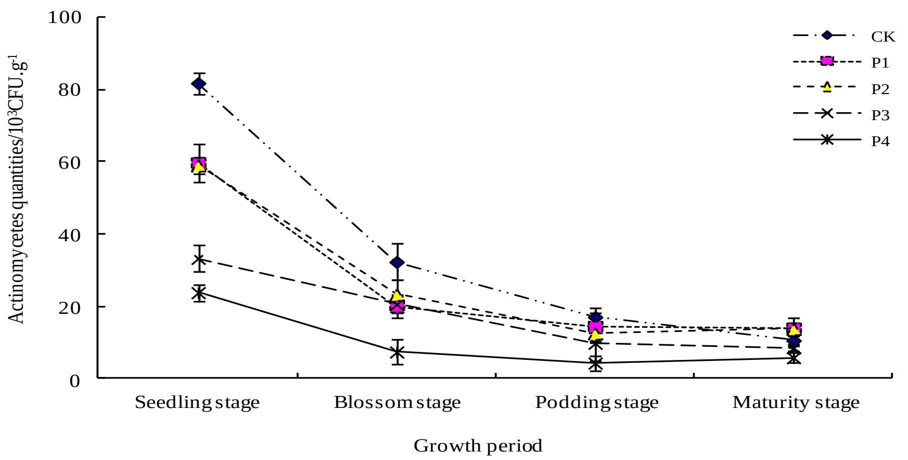


Figure 5. Effect of vanillic acid on the number of actinomycetes in rhizosphere soil in pot culture

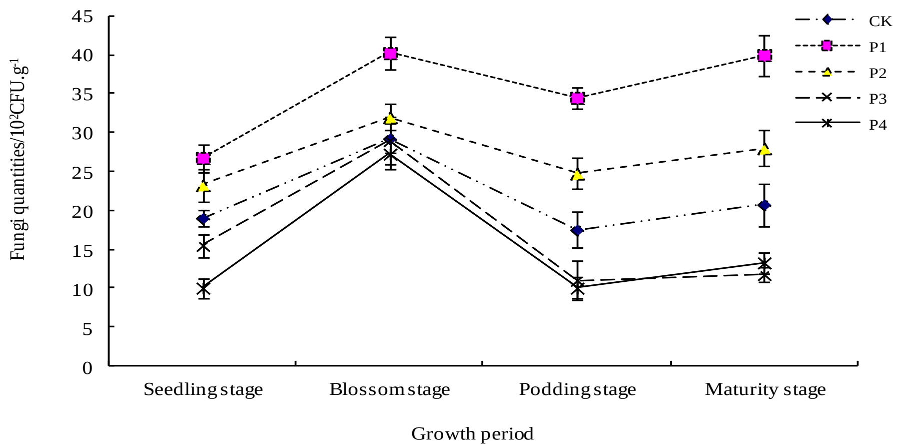


Figure 6. Effects of Vanillic acid on rhizosphere soil Fungi in pot culture

Similarly, VA also affected the fungal number in peanut rhizosphere soil (Fig. 6). The number of rhizosphere fungi varied from seedling stage to maturity. Their numbers increased initially but decreased subsequently**.** The fungal numbers in P1 and P2 treatments was higher than in control during the whole growth periods, while, the numbers in P3 and P4 were always lower than in control during the whole growth period. These results suggest that lower concentration of vanillic acid promoted the growth of rhizosphere fungi, while higher concentrations were inhibitory.

## DISCUSSION

The mechanisms involved in continuous cropping problem are very complex. The previous studies have shown that the accumulation of autotoxins such as phenolic acids is one of the factors causing Continuous Cropping problem in peanut cultivation (5,12,18,20). Many studies regarding autotoxins in crops (Cucumber, wheat and soybean) have shown that these chemicals inhibits the plant growth by affecting the plant metabolic processes (1,41,44). In recent years, research on autotoxicity in peanuts has been done but mechanism of autotoxicity has not been studied. Liu *et al.* (22) reported that the peanut root exudates collected by the continuous trapping system, had autotoxic potential. They reported that the autotoxins from roots inhibited the growth of peanut seedlings by inhibiting the radicle elongation and destroying the membrane structure. Li *et al.* (19) also identified the type and quantity of phenolic acids from the Continuous Cropping peanuts fields and their impact on the growth of peanut plants. Their results showed that 3-phenolic acids (p-hydroxybenzoic acid, vanillic acid and coumalic acid) present in peanut soil get accumulated in continuously cropped peanut soils**.** These phenolics not only inhibited the growth of seedlings but also increased the incidence of peanut pathogens.

In our earlier studies**,** we reported the presence of 4- phenolic acids (p-hydroxybenzoic acid, vanillic acid, coumaric acid and coumarin) in the rhizospheric soil of peanuts. The concentration of these phenolics was higher than other phenolic acids and their concentrations increased with the number of years of continuous cropping (13). After 4-years cropping, the vanillic acid and coumarin contents were 0.289 $\mu g.g^{-1}$ dry soil and 0.025 $\mu g.g^{-1}$ dry soil, respectively. However, no systematic research was conducted to assess the toxic effects of these phenolic acids on Peanuts. Therefore, in this study, we evaluated the toxicity of VA and its effects on Peanut seed germination and seedling growth. We found that vanillic acid inhibited the peanut seed germination and seedling growth and this inhibition was concentration dependent.

It is well known that the root system of plants is the most active organ involved in absorption and synthesis. Our results showed that VA had strong inhibitory effects on the root activity of peanut seedlings, the content of leaf chlorophyll and the rate of photosynthesis and the inhibition increased with increasing concentration of VA. On the contrary, vanillic acid enhanced the content of MDA leaves and intercellular $CO_2$ concentration. It appears that the inhibition of photosynthesis leads to an accumulation of $CO_2$. These results showed that phenolic acids not only inhibited the growth of underground parts, but also affected the above ground plant development (2,9,23,32).

Soil microorganisms are crucial for the sustainability and function of soil-based ecosystems (7,33,34). Recent, studies on the effects of phenolic acids (coumaric and vanillic acid) on soil microbial communities have shown that their addition to the soil can affect the soil microbial population and also contributes to the development of soil borne diseases (14,26,29,47). In this study, although not very quantitative, the effect of adding VA on the variation of Peanut rhizosphere microflora was examined in pot culture studies. It was found that VA decreased the number of bacteria and actinomycetes in the peanut rhizosphere but it promoted the growth of rhizosphere fungi at low concentration, suggesting that this phenolic acid may have a role in stimulating the plant pathogenic fungi at low concentration. Generally, during the whole growth period, the bacterial numbers in the rhizosphere soil showed an increasing trend but their numbers in all treatments were lower than in control. The reason perhaps is that VA increases the diversity of bacteria in the soil leading to a decline in the number of specific bacteria. Similar results have been reported by Liu *et al*. and Zhou *et al* (21,48). However, in our studies we assessed the microbiological status by conventional procedures which give an indication of only those microbes which can be cultured by routine methods. Considering that these methods give an indication of only a percent of the whole population, great emphasis cannot be placed on these results at present. Precise estimation of the VA on the rhizosphere microbial populations will have to wait the use of high-throughput sequencing of 16SrRNA gene fragments studies. (21,42,43,46).

## CONCLUSIONS

The VA, (one of the four phenolics found in the root exudates and soils in which peanut plants were grown), had toxic effects on the peanut seed germination and adversely affected the growth parameters. Seed germination and other plant growth characteristics were significantly affected by vanillic acid at concentration > 0.01 $mM.L^{-1}$. The VA also altered the rhizosphere microbial status, an additional factor in continuous cropping problem. The number of rhizosphere bacteria and actinomycetes were markedly reduced with an increase in vanillic acid concentration. Higher concentrations of VA also inhibited the rhizosphere fungi but lower concentrations were stimulatory.

## ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Grant No.31401948), The National Key Research and Development Program of China (2017YFD0200803) and National Peanut Industry Technology System Fund of China (CARS-14).

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