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# EFFECTS OF SIMULATED ACID RAIN ON PHYSIOLOGICAL CHARACTERISTICS AND ACTIVE INGREDIENT CONTENT OF *ASPARAGUS COCHINCHINENSIS* (LOUR.). MERR.

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**Abstract**

The effects of simulated acid rain at pH 2.0, 3.0, 4.0, 5.6 and 6.8(control) on the morphological characteristics, chlorophyll content, resistance physiology and active ingredient content of *A. cochinchinensis* cultured in pots were studied so as to explore the sensitivity and tolerability of *A. cochinchinensis* to acid rain with the hope to provide the theoretical basis for the cultivation of *A. cochinchinensis*. The results showed that 1) compared to the control, acid rain with pH 5.6 did not significantly affect the shape and color of the leaves as well as the contents of amino acids and soluble sugar in tuber roots of *A. cochinchinensis*, but significantly enhanced the activities of superoxide dismutase (SOD) and peroxidase (POD), and 2) with the increase of acid rain stress (pH≤3), the damage degree of leaves and the content of malondialdehyde (MDA) in leaves gradually increased, but the photosynthetic pigment content, stem length, root weight increment, POD and SOD activities, as well as contents of amino acids, soluble sugar and saponin in tuber roots all decreased. The experiments indicated that 1) acid rain significantly affected the physiological characteristics and active ingredients accumulation of *A. cochinchinensis*, 2) *A. cochinchinensis* showed a certain resistance to acid rain, 3) pH ≤ 3 is the critical point (threshold) for acid rain to damage *A. cochinchinensis*, and 4) artificial cultivation of *A. cochinchinensis* should avoid medium and strong acid rain pollution.

**Key words**: Simulated acid rain, *A. cochinchinensis*, Physiological characteristics, Active ingredients.

## Introduction

Acid rain refers to rainwater with pH< 5.6 and other forms of acid precipitation such as snow, hail and fog and is also called acid precipitation (Anon., 2005). Southwest China has one of the three major acid rain regions in the world. These regions are mainly distributed in the vast area south of the Yangtze River. In recent years, the contents of sulfur and nitrogen oxides in the atmosphere have increased significantly, resulting in increase in acid rain affected areas year by year and increasingly serious damages to the regions.

Acid rain could directly or indirectly impact plant growth and development, such as inhibit seed germination, affect seedling growth and development (Tong & Zhang, 2014; Ramlall *et al.*, 2015), alter activities of resistance enzymes, promote biochemical process disorders (Wyrwicka & Sklodowska, 2007; Liu & Liu, 2011), hinder plants photosynthesis and affect crop yield and quality (Velikova *et al.*, 1999; Khalid *et al.*, 2013; Liu *et al.*, 2015). For example, Du *et al.*, (2017) analyzed data from the published literature on the effect of simulated acid rain on 67 terrestrial plants in China. They found that acid rain substantially reduced leaf chlorophyll content by 6.71% per pH unit across the recorded plant species and directly damage foliage, causing production reduction and economic loss. Chen *et al*., (2013) studied the influence of simulated acid rain on the seedlings of *Liquidambar formosana* and *Schima superba*. They found that acid rain could cause leaf necrosis, inhibit photosynthesis, induce superoxide radical and hydrogen peroxide generation, aggravate membrane lipid peroxidation, and change antioxidant enzyme activities. Lee *et al*., (1981) revealed the response patterns of major crops in USA to sulfuric acid rain. They found that acid rain markedly inhibited the yield of 5 crops (radish, beet, carrot, mustard greens, broccoli), stimulated growth of 6 crops (tomato, green pepper, strawberry, alfalfa, orchardgrass, timothy), and ambiguously affected the yield of 1 crop (potato). In a word, acid rain has significantly different effects on different plants. Overall, previous studies are mostly focused on the effects of simulated acid rain on grain crops, economic crops and some forest species. The effects of simulated acid rain on medicinal plants are not well explored.

*Asparagus cochinchinensis* belongs to genus Asparagus of family Liliaceae. Its dry tuber roots contain many active ingredients such as amino acids, polysaccharides and saponins, and its main parts are used as components of daily meals and traditional Chinese medicines for treatment of diabetes, bronchitis, whooping cough, diphtheria, dry stool and tuberculosis (Anon., 2015). With the demand of medicine market increasing, the wild resource of *A. cochinchinensis* has gradually depleted due to predatory mining. Therefore, *A. cochinchinensis* has become a wild medicinal herb species need to be specially protected. Currently, wild *A. cochinchinensis* resources have been unable to meet the market demand, so artificial cultivation and rational development and utilization of the germplasm resources have become the most effective way to protect *A. cochinchinensis*. China's *A. cochinchinensis* resources are mainly distributed in about 700 counties (cities) in Jiangsu, Zhejiang, Jiangxi, Hunan, Hubei, Sichuan, Guizhou, Yunnan, Guangxi, Guangdong and Fujian provinces (Anon., 1995). Among them, the south Yangtze River is the main producing area. In other words, China's acid rain area basically overlaps with the main distribution area of *A. cochinchinensis*. Therefore, studies on the effects of acid rain on *A. cochinchinensis* cultivation are very necessary.

In this study, the effects of acid rain on the growth and development of *A. cochinchinensis* were explored by simulating the effects of different concentrations of acid rain on the agronomic traits, resistant physiology and active ingredients content of *A. cochinchinensis* so as to provide a theoretical basis for cultivation and promotion of high yield *A. cochinchinensis* cultivars and protection of wild *A. cochinchinensis* germplasm resources.

## Materials and Methods

The experiment was carried out in the greenhouse of Huaihua University. The experimental material was *A. cochinchinensis* planted in Huaihua University Botanical Garden (identified by Professor Hanyuan Zeng of Huaihua University). In March 2016, after removal of the upper parts, *A. cochinchinensis* plants, which were in uniform and good growth condition, were planted in pH 6.85 loam in 25 pots, which had upper diameter of 21 cm, lower diameter of 14 cm and height of 19 cm, with one plant per pot. These plants were randomly divided into 5 groups with 5 plants per group. During the rejuvenation period, plants were regularly subjected to tap water irrigation, fertilization and weed removal. The simulated acid rain treatment was carried out in mid-April 2016.

The simulated acid rain stock solution was prepared by mixing $H_2SO_4$ and $HNO_3$ at 8:1 (v/v) (Zhao, 2014) and diluted using distill water to working acid rain solution with pH 2.0, 3.0, 4.0 and 5.6, respectively. In addition, distill water with pH 6.8 was used as control. The acid rain treatment was performed using the spray method. The amount of sprayed acid rain was 40 ml per 3 days per pot for 60 days, which was determined according to the average monthly precipitation in Huaihua City (data from the Huaihua Meteorological Bureau). Samples were collected 3 days after the last spraying to measure the related physiological indexes.

**Observation of plant morphology:** Agronomic traits such as leaf color, shape and shedding were observed and used to evaluate the damage degree of acid rain to the plants. In addition, agronomic traits such as stem length and fresh weight of tuber roots were measured before and after the treatment and used to calculate the growth mass of stem and roots.

**Measurement of leaf chlorophyll content:** The leaf chlorophyll content was determined using spectrophotometry (DU-800, USA) according to the method of Arnon (1949) after 95% ethanol extraction.

**Measurement of MDA content and SOD, POD activities:** The content of malondialdehyde (MDA) was determined by thiobarbituric acid method (TBA). MDA in lipid degradation products can be condensed with TBA to form a red product, and its maximum absorption at 532 nm was used to calculate the content of MDA (Dhindsa *et al.*, 1981).

Extraction and assay of superoxide dismutase (SOD) were based on the method of Beauchamp and Fridovich (Ding *et al.*, 2008) by measuring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. One unit of SOD is defined as the amount required for inhibiting photo-reduction of NBT by 50%. The specific activity of SOD was expressed as unit per mg SOD protein.

The activity of peroxidase (POD) was determined by guaiacol method. Under the catalysis of POD, $H_2O_2$ can oxidase guaiacol to tea brown products. This product has a maximum absorption value at 470 nm, so the activity of POD can be determined by measuring its absorbance at 470 nm (Kochhar *et al.*, 1979).

**Measurement of active ingredients content in tuber roots:** The contents of free amino acids, soluble sugars and total saponins in *A. cochinchinensis* roots were determined by ninhydrin colorimetry, anthrone colorimetry, vanillin-glacial acetic acid-perchloric acid colorimetric method, respectively.

**Statistical analysis:** The experimental data was presented as the average ± the standard deviation. And Duncan multiple comparisons were conducted among treatments using SPSS 13.0 software, and the significance level was set at 5% level.

## Results

**The effects of simulated acid rain on morphology of *A. cochinchinensis*:** Differences in morphological characteristics of *A. cochinchinensis* after treatment with acid rain solution with different pH are shown in Table 1. Overall, plants treated with pH 5.6 acid rain and plants in control group showed similar agronomic traits such as bright green leaves, normal leaf shape and little shedding. After treated with pH 4.0 acid rain solution, although most leaves remained green, some appeared to be curled and shedding, indicating that the plants were affected, but could still grow normally. With the pH further decreasing, the damage degree of plants increased, as the leaf color changed from green to yellow and from yellow to white, and the leaves were curled and withered.

The stem length of *A. cochinchinensis* was positively correlated with the pH of acid rain, and increased with pH of acid rain decreasing. The stem length was the highest, reaching 6.77 cm in the control group. In addition, the change of fresh root weight showed a similar trend, with maximum increase of 2.10 g in the control group. By comparison, *A. cochinchinensis* treated with pH 2.0 and 3.0 acid rain solution have roots with various hollow degree and decreased fresh weight. In addition, the rooting number of *A. cochinchinensis* was significantly different under different acid rain treatments, showing the highest at pH 4.0, and a decreasing trend with pH further lowering.

**Effects of simulated acid rain on leaf chlorophyll content of *A. cochinchinensis*:** As an important pigment in photosynthesis, chlorophyll can convert captured light energy into chemical energy. But its content is affected by various stress conditions. As shown in Table 2, chlorophyll a, chlorophyll b and total chlorophyll content of *A. cochinchinensis* decreased with the decrease of acid rain pH. The total chlorophyll content of *A. cochinchinensis* was 3.86 mg/g in the control group, but decreased to 2.89 and 1.11 mg/g in pH 3.0 and pH 2.0 acid rain treatment groups, consistent with the morphological observation.

**Table 1. Effect of simulated acid rain on morphological characteristics of *A. cochinchinensis*.**

| Treatment | Leaf color | Leaf shape | Leaf shedding | Stem length (cm) | Root weight increment (g FW) | Rooting number |
|---|---|---|---|---|---|---|
| CK | Bright green | Normal | Little | 6.77 ± 0.52a | 2.10 ± 0.19a | 4.00 ± 0.31b |
| pH 5.6 | Bright green | Normal | Little | 5.36 ± 0.42b | 0.98 ± 0.12b | 4.40 ± 0.41b |
| pH 4.0 | Green | Partial curled | Less | 4.29 ± 0.33c | 0.05 ± 0.03c | 5.80 ± 0.48a |
| pH 3.0 | Yellow | Curled, withered | Partial | 3.33 ± 0.40d | -0.19 ± 0.06c | 3.20 ± 0.37c |
| pH 2.0 | Yellowish white | Curled, withered | More | 3.2 ± 0.11d | -1.35 ± 0.11d | 1.60 ± 0.22d |

Note: Different normal letters in the same columns indicate significant differences at 5% level, the same as below

**Table 2. Effect of simulated acid rain on chlorophyll content in leaves of *A. cochinchinensis*.**

| Treatment | Chlorophyll a (mg/g FW) | Chlorophyll b (mg/g FW) | Total Chlorophyll (mg/g FW) |
|---|---|---|---|
| CK | 2.78 ± 0.18a | 1.07 ± 0.07a | 3.86 ± 0.26a |
| pH5.6 | 2.63 ± 0.38a | 1.04 ± 0.16a | 3.67 ± 0.54a |
| pH 4.0 | 2.52 ± 0.41a | 0.93 ± 0.28ab | 3.46 ± 0.68a |
| pH 3.0 | 2.13 ± 0.11b | 0.76 ± 0.07b | 2.89 ± 0.18b |
| pH 2.0 | 0.78 ± 0.13c | 0.33 ± 0.05c | 1.11 ± 0.18c |

**Effects of simulated acid rain on MDA content and SOD, POD activities in leaves of *A. cochinchinensis*:** Plant organs in the aging or stress environments tend to undergo membrane lipid peroxidation, producing malondialdehyde (MDA). Thus, MDA level could reflect the degree of cell membrane damage. It can be seen from Fig. 1 that MDA content in *A. cochinchinensis* leaves increased gradually with acid rain pH decreasing and the difference in MDA level between the control and treatment groups also gradually increased with acid rain pH decreasing, showing the highest difference of 2.78-fold between control and pH 2.0 acid rain treatment groups. The results showed that cell membrane damage was severe, in consistence with the apparent damage caused by acid rain to *A. Cochinchinensis*.

SOD and POD are important active oxygen scavenging enzymes in organisms. They play important roles in scavenging superoxide radicals and reducing lipid peroxidation and membrane damage. As shown in Fig. 2, compared with that of the control, SOD activity in *A. cochinchinensis* increased under pH 5.6 and pH 4.0 acid rain treatments, but decreased under pH 3.0 and pH 2.0 acid rain treatments. Similarly to that of SOD, POD activity also showed a trend of decrease after increase with acid rain pH decreasing. Together, these results showed that the activities of the protective enzymes and pH resistance of *A. cochinchinensis* increased under low acid rain stress, suggesting that *A. cochinchinensis* could adapt to the acid rain stress to a certain extent. But the activities of these enzymes decreased significantly under strong acid stress, indicating that it caused irreversible damages to the plant.

**Effects of simulated acid rain on active ingredient contents of *A. cochinchinensis*:** Free amino acid content in the root of *A. cochinchinensis* is significantly affected by acid rain pH (Fig. 3), showing an overall trend of gradual decrease with acid rain pH decreasing. The content of amino acids was the highest of 2.58% in the control group and the lowest in the pH 2.0 acid rain treatment group. The soluble sugar content was the highest of 7.28% in the pH 5.6 acid rain treatment group and decreased with pH decreasing. The content of saponin had a similar trend to that of free amino acid content, showing the highest of 3.21% in the control and the lowest in pH 2.0 acid rain treatment group, which was only 23.36% of that in the control group. Overall, the results showed that acid rain stress had a great effect on the active ingredient content of *A. cochinchinensis*, and excessive stress was not conducive to the accumulation of effective components.

## Discussion

**Effects of acid rain on physiological characteristics of *A. cochinchinensis*:** Previous studies have shown that acid rain has a direct impact on plant growth and development. It can affect plant growth, induce visible damage to leaves, change plant enzyme activities and influence plant photosynthetic and physiological functions (Lee & Weber, 1979; Khalid *et al.*, 2017). Our results showed that *A. cochinchinensis* plant subjected to acid rain of pH 5.6 had few shedding leaves and similar leaf color and morphological characteristics to those of *A. cochinchinensis* in the control group. But with increase of acid rain stress, especially under the strong acid rain (pH≤3), the leaves of *A. cochinchinensis* were seriously damaged, showing obvious curling and wilting. In addition, chlorophyll content gradually decreased with acid rain stress increasing, and the leaves gradually changed from bright green to light green, even yellow. The main mechanism of acid rain damaging plant is that the acid rain reduces activities of membrane protective enzymes, imbalances active oxygen metabolism, and induces membrane peroxidation (Wang *et al.*, 2013). Our results showed that MDA content in *A. cochinchinensis* leaves increased with acid rain stress increasing, while the contents of POD and SOD presented a trend of increase before decrease with acid rain pH decreasing, indicating membrane peroxidation was significantly increased in *A. chinchinensis* subjected to acid rain treatment. At low acid rain stress, plants protect themselves by increasing activities of POD, SOD and other antioxidant enzymes to adapt to acid rain stress. However, further aggravation of acid rain stress reduces the abilities of POD, SOD and other antioxidant enzymes to remove reactive oxygen species, leading to the further accumulation of reactive oxygen species and eventual damages to cell membrane function and chloroplast structure, and reduction of chlorophyll content (Wyrwicka & Sklodowska, 2006; Tao *et al.*, 2014). Chlorophyll, as the main pigment of plant photosynthesis, directly affects photosynthesis intensity and plant growth and development. Our results also showed that with the decrease of acid rain pH, the stem length and root weight increment of *A. cochinchinensis* decreased. In addition, the roots of *A. cochinchinensis* appeared to become hollow at different degrees under the strong acid rain stress, suggesting that acid rain significantly affected the growth and development of *A. cochinchinensis*.

Severe acid stress seriously damaged *A. cochinchinensis* leaves, destroyed its photosynthetic structure, decreased its photosynthetic pigment content, and reduced its photosynthetic products synthesis and transportation to the roots, as manifested as weak and small plant as well as appearance of hollow roots. However, the root number of *A. cochinchinensis* showed a trend of decrease after increase with acid rain pH decreasing, reaching its peak at pH 4.0. These phenomena are probably due to that plants need to absorb more nutrients under acid rain stress by promoting the growth of new root, so as to adapt to the adverse environment (Fu *et al.*, 2006).

**Effects of acid rain on the content of active ingredient in *A. cochinchinensis*:** Numerous studies have shown that acid rain can cause physiological metabolic disorders, damage leaf photosynthetic system and decrease photosynthetic capacity and products, resulting in reduced crop yields and quality (Odiyi & Bamidele, 2014). Khan *et al*., (2004) found that the contents of sugar, nitrogen and protein in potted *Phaseolus vulgaris* decreased with the increase of acid rain acidity, resulting in its quality decrease. Evans *et al*., (1981) showed that acid rain stress could significantly decrease protein content of Amsoy soybean variety. Qi *et al*., (2006) found that the contents of chlorogenic acid, aucubin and total flavonoids in *Eucommia ulmoides* Oliv. leaves decreased with the decrease of acid rain pH. In this study, we showed that the contents of amino acids and saponin in the root of *A. cochinchinensis* decreased with the increase of acid rain stress, and the soluble sugar content was the highest at pH 5.6 and decreased with the decrease of acid rain pH, indicating that acid rain stress was not conducive to accumulation of active ingredients in *A. cochinchinensis*. Previous study (Zhang, 2012) showed that in order to adapt to the environment, plants will take the initiative to accumulate soluble sugar to protect their own systems, and $SO_4^{2-}$ and $NO_3^-$ in acid rain could participate plant carbohydrate metabolism and promote carbohydrate synthesis. However, strong acid rain stress could seriously damage plants, decrease their photosynthetic capacity and inhibit the synthesis of soluble sugars, amino acids, saponins and other metabolites.

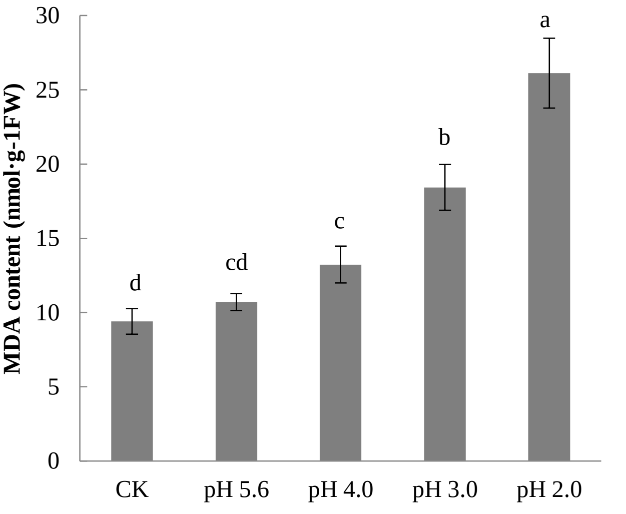


Fig. 1. Effect of simulated acid rain on MDA content in leaves of *A. Cochinchinensis*.

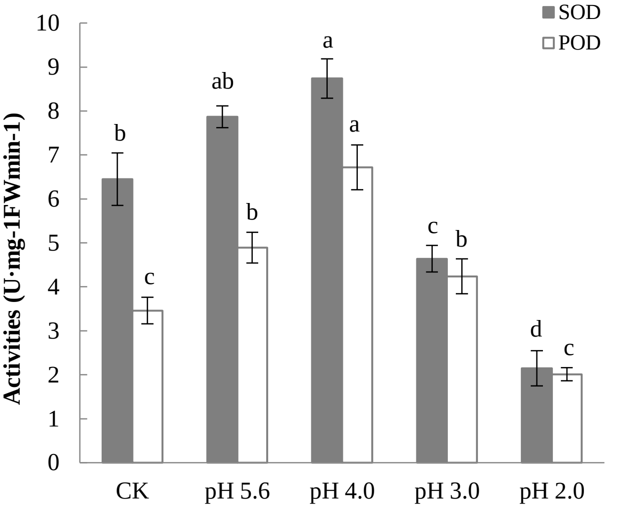


Fig. 2. Effect of simulated acid rain on SOD and POD activities in leaves of *A. cochinchinensis*.

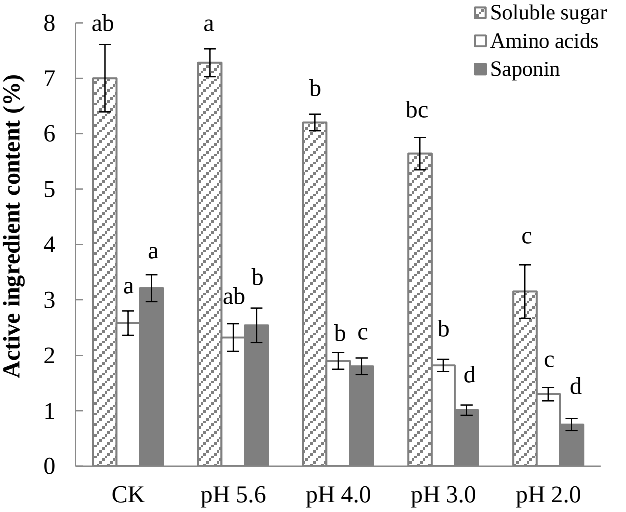


Fig. 3. Effect of simulated acid rain on active ingredient content in roots of *A. cochinchinensis*.

## Conclusions

In summary, acid rain has significant impact on physiological characteristics and active ingredient accumulation of *A. cochinchinensis*. At low acid rain stress (pH 5.6), the shape and color of *A. cochinchinensis leaves* were not significantly different from those in the control group, while the activities of protective enzymes such as SOD and POD were increased, showing a certain degree of protection, and the contents of amino acids and soluble sugars in the roots were not significantly higher than those in the control, indicating that in the areas that acid rain is not very serious, artificial cultivation of *A. cochinchinensis* is feasible. However, increased acid rain stress (pH≤3), could make the leaves of *A. cochinchinensis* yellow and shed, increase the content of MDA, decrease the contents of photosynthetic pigments and activities of SOD and POD, reduce plants growth and inhibit active medicinal ingredients accumulation. Therefore, the comprehensive physiological indicators indicated that pH ≤ 3 is the critical point (or threshold) of acid rain to have significant damages to *A. cochinchinensis*. Thus, medium and strong acid rain stress should be avoided in artificial cultivation of *A. cochinchinensis*.

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