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酚顿试剂对竹林土壤中酚类化合物的降解作用

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摘要: 酚类化合物是农业和生态系统中主要的化感物质之一, 大量积累在土壤中, 抑制作物和林下植物生长, 导致农作物减产、连作障碍和自然生态环境破坏。用过氧化氢(H_2O_2)与硫酸亚铁(Fe^{2+})所组成的酚顿试剂研究了化学氧化法对化感物质(对香豆酸、对羟基苯甲酸和胡桃醌)、竹林土壤提取物及竹林土壤中对香豆酸的降解作用。过氧化氢与硫酸亚铁的摩尔比为 15:1 的酚顿试剂, 过氧化氢与酚类物质浓度比为 8:1 时, 对酚类物质的降解效率最高。以 8×10^{-3} mol/L (0.028%) 的 H_2O_2, 5.4×10^{-4} mol/L (0.075%) 的 $FeSO_4$ 和 10^{-3} mol/L 的酚类物质组成的反应体系中, 反应 10 min 和 30 min 后, 对香豆酸的降解率分别为 55%和 74%; 对羟基苯甲酸的降解率在 10 min 时达 90%以上; 而胡桃醌在 10 min 时已经完全被降解。酚顿试剂处理土壤酚类提取物时, 可使其中主要的化感物质对香豆酸降解 75%。用含 0.1%和 1% H_2O_2 的酚顿试剂处理竹 (*Bambusa chungii*) 林土壤, 土壤对香豆酸的降解率分别为 32%和 37%。竹林土壤中存在过氧化氢酶和过氧化物酶活性, 但没有检测到超氧化物歧化酶活性。土壤中的过氧化氢酶和过氧化物酶可能迅速分解外加的过氧化氢, 一方面缩短过氧化氢处理的作用时间和降低降解效率, 另一方面可分解过剩的过氧化氢。这说明酚顿试剂是降解土壤和培养液中有害化感物质的有效化学氧化剂。

关键词: 化感物质降解; 酚类物质; 酚顿试剂; 土壤; 竹林
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Degradation of Phenolic Allelochemicals in Bamboo Soil by Fenton's Reagent

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Abstract: Phenolic compounds are toxic allelochemicals widely distributed in agriculture and natural ecosystems. They accumulate largely in plant soils, inhibit plant growth and pollute environments. Oxidative degradation of phenolic allelochemicals by Fenton's reagent was studied under natural conditions. In a 1.0 ml reaction solution containing 8×10^{-3} mol/L (0.028%) of hydrogen peroxide (H_2O_2), 5.4×10^{-4} mol/L (0.075%) of $FeSO_4$, and 10^{-3} mol/L of phenolics or 160 μg ml^{-1} of soil extracts, *p*-coumaric acid was oxidized by 55% and 74% in 10 and 30 minutes, respectively; *p*-hydroxybenzoic acid was oxidized by 90% within 10 minutes; juglone was completely oxidized within 10 minutes. *p*-Coumaric acid, the main allelochemical in extracts of soil beneath forest of bamboo (*Bambusa chungii*), was oxidized by 75% in 2 h. Direct application of 0.1% or 1% of H_2O_2 of Fenton's reagent to the soil from bamboo forest resulted in a 32% or 37% degradation of *p*-coumaric acid in the soil. Activities of antioxidant enzymes of catalase and peroxidase, but no superoxide dismutase, were detected in the bamboo soil. These enzymes may decompose H_2O_2 employed to soil and reduced its function in soil. Meanwhile, they are

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responsible for decomposition of surplus H_2O_2 in the soil. Results indicated that Fenton's reagent might be a potential reagent for degradation of toxic allelochemcials in plant soils and cultural solutions.

Key words: Allelochemical degradation; Phenolic compounds; Fenton's reagent; Plant soil; *Bambusa chungii*

Phenolic allelochemicals have been reported to play a major role in allelopathy in a wide range of plant species [1-3]. They can be released into soil environments as root exudates, leaf leachates, and products of plant tissue decompose, or released from bound forms and transformation by microorganisms. These released chemicals accumulated highly in soils, producing significant effects on growth of neighboring plants[3-4]. Inhibitory or toxic effects of allelochemicals on germination and/or plant growth are well documented in many species[5-12].

The degradation or inactivation of phenolics from soil is a result of ionization, oxidation, polymerization, sorption onto soil particles, seed and root uptake, and transformation and utilization by microorganisms [3,13]. Concentrations of phenolics in soils are determined by their dynamic balance between input and output in soils. Although it was hypothesized that phenolics are readily metabolized by soil and soil microorganisms that allelopathic by phenolic acids are highly unlikely[3], phenolic acids was reported to be accumulated in high amounts in soils of many crops and forestry plants, and inhibit growth of their neighboring plants[5-9,14-17].

Hydrogen peroxide (H_2O_2) together with a metal iron catalyst (usually Fe^{2+}), commonly called Fenton's reagent, was capable of oxidizing many organic compounds to low molecular substances and eventually to carbon dioxide and water. Oxidation of organic compounds catalyzed by the Fenton's reagent could complete in a short period of time under natural conditions[18]. Their reaction products are not toxic to lives and environments. Recently, Fenton's reagent has been used as a chemical oxidant for degradation of phenolic compounds in water industry and wastewater treatment[18-20]. Degradation of toxic allelochemicals in plant soil by the Fenton's reagent has not been reported.

Bamboo is one of the widely distributed plants in the World. It is well known that in most cases, there were almost no grasses growing beneath and near bamboo forests. Tsai and Young identified eleven phenolic compounds from the soil beneath bamboo, *Denrocalamus latiflorus* Munro. *p*-Coumaric acid was the main inhibitory component in the soil[16]. In this research, oxidative degradation of phenolic allelochemicals catalyzed by the Fenton's reagent was studied in soil extracts and soils beneath forests of a bamboo species, *Bambusa chungii* McClure. Results indicated that Fenton's reagent is a potential reagent for degradation of toxic allelochemcials in plant soils and cultural solutions.

1 Materials and methods

1.1 Chemical reagents

Chemical compounds we used are Sigma products (*p*-coumaric and *p*-hydroxybenzoic acids, and juglone) or analytic grade from local companies. HPLC solvents are products of Burdick & Jackson Inc.

1.2 Fenton's reagent

Fenton's reagent consists of H_2O_2 and Fe^{2+} ($FeSO_4$) in a certain molar ratio. Based on Peres's report[19], the oxidative efficiency of different ratios of H_2O_2 to Fe^{2+} was tested. It was found that a ratio of 15 : 1 of H_2O_2 : Fe^{2+} in molar concentrations was the most effective combination for the degradation of phenolic substances, which was used for all degradation tests.

1.3 Soil samples

Soil samples were obtained from the 5 to 10 cm layer from soil surface at the center area of a natural forest of bamboo (*Bambusa chungii*) at Tianhe Public Park in Guangzhou City, China. Soil samples were used immediately after sampling or stored in a -20℃ freezer for later use.

1.4 Soil treatment with H_2O_2

As wet soil contained lots of stones and debris, fresh soil was dried in an electric dryer at 70℃ for 4 h. The dried soil was passed through a 0.9 μm sieve. For each treatment, 25 ml of the Fenton's reagent (or distill water for control) containing 0.1% or 1.0% H_2O_2 was added to 50 g soil powder in a glass cup and incubated at room temperature for 4 h, stired thoroughly with glass bar every half hour. After treatment, soil samples were dried again in a 70℃ electric dryer for 4 h. The dried soil was used for extraction and analysis of allelochemicals.

1.5 Extraction of phenolic allelochemicals from soil

Extraction procedure for phenolic compounds from soil was based on our previous method[10] with some modification. Soil powder of 1 g was extracted with 1 ml extraction solution (1 mol/L NaOH) in a 2 ml plastic eppendorf tube at 40℃ for 4 h, vortexed and sonicated 5 min every half hour. Extraction mixtures were centrifuged at 2 300×g for 10 min. Supernatant of 500 μl was carefully pipeted to a new eppendorf tube without disturbing the pellet. The extraction solution was adjusted to pH 2.0 by adding 108 μl of 5 mol/L HCl, fractionated with 600 μl of ethyl acetate for 30 min, and centrifuged at 2 300×g for 10 min. The ethyl acetate fraction of 350 μl was removed to a fresh tube and dried at room temperature (overnight). Each dried samples were redisolved in 100 μl 50% methanol and used for HPLC analysis.

Recovery tests were performed by adding standard compound (*p*-coumaric acid) into the soil, followed by the extraction and HPLC analysis described previously. Solution containing 0.2 mg of standard compound of *p*-coumaric acid was added to 1 g of soils and total *p*-coumaric acid in the soils was extracted and analyzed by HPLC. A recovery rate of (72±1.8)% was detected in three independent repeats through this extraction and analysis method. The recovery rate seems to be low, possibly because of a strong sorption of soil particles to the compounds.

1.6 Degradation tests of phenolic compounds and soil extracts by Fenton's reagent

As we know, a molar ratio of H_2O_2 : phenolics of 8 : 1 was effective in the degradation of the phenolic compounds. Reaction rate or final concentrations of substrates will not be affected by the concentrations of over 8 : 1 of H_2O_2 to phenolics (data not shown). The reaction system contained 8×10^{-3} mol/L H_2O_2, 5.4×10^{-4} mol/L $FeSO_4$ and 10^{-3} mol/L allelochemicals (or 160 μg ml^{-1} of soil extracts) in a total volume of 1 ml in eppendorf tubes. Reaction was performed at room temperature (28℃) and started by adding the last reagent of H_2O_2 solution. Concentrations of allelochemicals in the reaction solutions were analyzed by HPLC (injection of 10 μl reaction solution) at different reaction time.

Quantitative and qualitative analysis of chemicals and soil extracts were carried out on a HPLC system (Dalian Elite Analytical Instrument Co., Ltd.), using a Hypersil ODS column (4.6 × 200 mm, 5 μm) and a UV230+ detector. UV absorbance was detected at 285 nm for *p*-coumaric acid and 254 nm for other compounds. An EC2000/EC2003 Spectrum Data Analyzer was used for data recording and analysis. Mobile phases used were the same as described before[10-11], which consists of solution A (acetonitrile : 1% acetic acid, 20 : 80, v/v) and solution B (acetonitrile : 1% acetic acid, 40 : 60, v/v). Solution A was used for the analysis of standard compounds. Gradient elution was used for analysis of soil extracts (0 to 10 min: solution A, 10 to 25 min: linear gradient from A to B, 25 to 40 min, solution B). Elution rate was 1.0 ml per minute. All samples were filtered through a 0.45 μm nylon filter before injected into HPLC. Contents of allelochemicals were determined by their correspondent peak areas. *p*-Coumaric acid in soil extracts of bamboo was identified by its retention time and its co-injection tests with standard compounds.

1.7 Determination of activities of antioxidant enzymes from soil

Three antioxidant enzymes of catalase, peroxidase

and superoxide dismutase (SOD) were extracted from the soil beneath the bamboo forest and their activities were assayed. Each soil sample was duplicates, one for analysis of enzyme activities in fresh soils, and the other was dried at 70℃ for 4 h and used for enzyme assays in dried soils and for determination of water content in fresh soils. One gram of soil was homogenized and extracted with 5 ml phosphate buffer (50 mmol/L, pH 6.4 for peroxidase and pH 7.6 for other two enzymes) for 20 min in ice bath. After centrifugation (1 500×g, 4℃, 10 min), the residues was extracted with 5 ml of the same phosphate buffer. The combined supernatant was stored at 4℃ and used for enzyme assays.

Catalase activity was determined by the method described by Roberge[23]. One unit of catalase activity means 1 μmol decrease of H_2O_2 per minute. Peroxidase activity was determined by its oxidation on guaiacol[24] and was expressed in $\triangle OD_{470}$ min^{-1} g^{-1} fresh soil. SOD activity was determined according to the method of Giannopolitis and Ries[25].

All experiments were repeated at least three times. Data presented are the means of three independent repeats±SE (standard error).

2 Results

2.1 Degradation of standard allelochemicals by Fenton's reagent

Three allelochemical compounds, *p*-coumaric and *p*-hydroxybenzoic acids and juglone were used to test the degradation efficiency by the Fenton's reagent. Concentrations of the three compounds were 10^{-3} mol/L, which are inhibitory concentrations for seed germination and plant growth[21-22]. *p*-Coumaric acid was oxidized by 55% and 74% after 10 and 30 min, respectively. *p*-Hydroxybenzoic acid was oxidized by more than 90% in 10 min. Then, the levels of *p*-coumaric acid and *p*-hydroxybenzoic acid

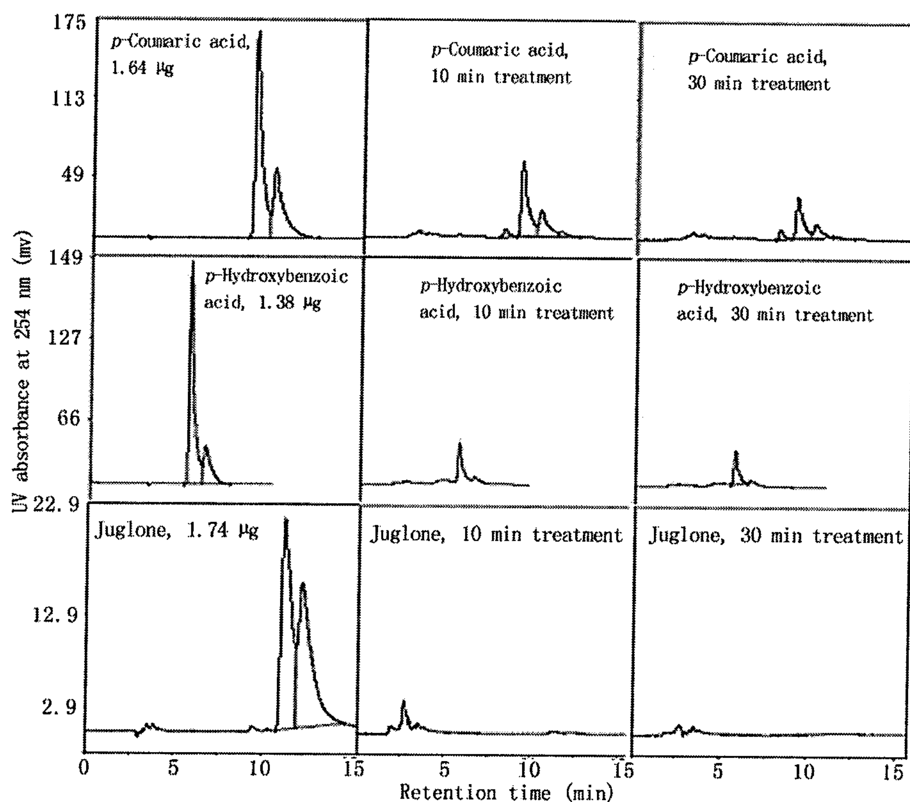

Fig. 1 HPLC analysis of *p*-coumaric acid (upper), *p*-hydroxybenzoic acid (middle) and juglone (lower) during oxidative degradation by Fenton's reagent

Left: before reaction; Middle: 10 min after reaction; Right: 30 min after reaction.

were kept for 2 h or longer. Juglone was almost completely degraded within 10 min (Fig. 1).

2.2 Degradation of phenolics in soil extracts treated by Fenton's reagent

Eight peaks (Fig. 2) could be distinguished in the HPLC chromatogram of soil extracts of the bamboo forest. The dominant peak was identified as *p*-coumaric acid. The other peaks were not identified. Soil phenolic extracts was diluted to a concentration of 160 μg ml^{-1}, which is equivalent to 10^{-3} mol/L of *p*-coumaric acid, in the reaction solutions.

Contents of *p*-coumaric acid in soil extracts were decreased by 44% and 52% in 10 and 30 min, respectively, and were subsequently decreased by 75% after 2 h. Except for one peak/substance right before *p*-coumaric acid in Fig. 2 which did not have a significant change in its size before and after H_2O_2 treatment, the rest peaks/substances had significant decreases after treated with Fenton's reagent.

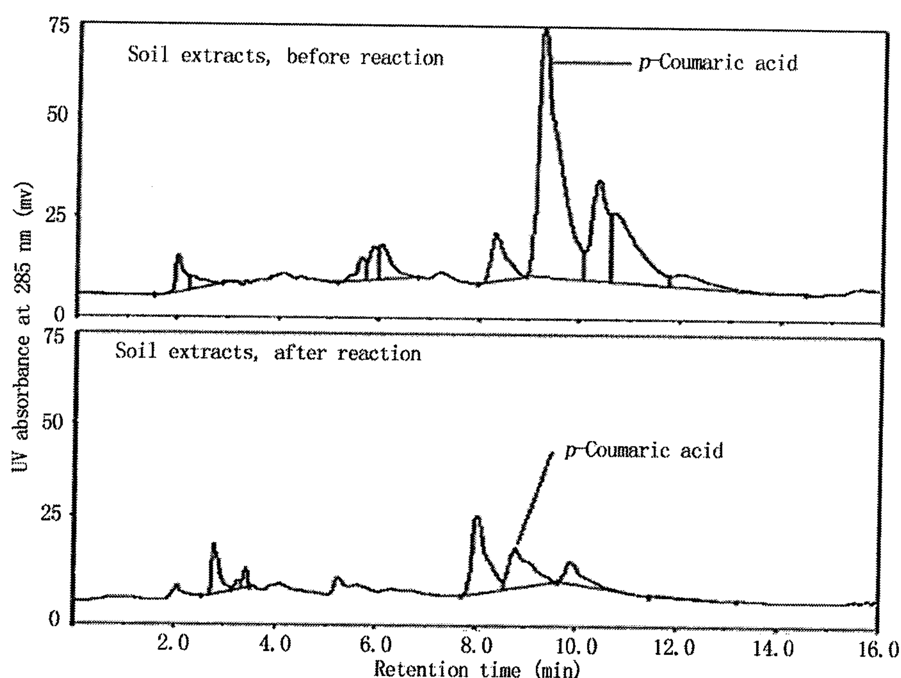

Fig. 2 HPLC chromatogram of soil extracts from soil beneath bamboo (*B. chungii*)
Up: Before treatment; Down: After two-hour Fenton's reagent treatment.

2.3 Degradation of phenolics in bamboo soils treated by Fenton's reagent

Both treatments with 0.1% and 1% of H_2O_2 of the Fenton's reagent for 4 h at room temperature resulted in a significant degradation of *p*-coumaric acid in soil beneath the bamboo forest. Soil contents of *p*-coumaric acid were decreased from 193.5 μg g^{-1} in the control treated with water to 132.6 or 121.8 μg g^{-1}, with a 31.5% or 37.1% degradation after treatments with 0.1% or 1% H_2O_2 of Fenton's reagent, respectively. The difference between the two treatments was not significant, which indicated that the Fenton's reagent functioned at a very low dose, and 0.1% of the reagent was almost a saturated concentration for the oxidation of soil phenolics.

2.4 Activities of H_2O_2 decomposing enzymes in soil

Three enzymes, catalase, peroxidase and SOD, are related to the decomposition of hydrogen peroxide in organisms. Their activities in soil samples of the bamboo forests were analyzed. Activities of catalase and peroxidase (Table 1) were detected in the soil.

Table 1 Enzyme activities of catalase, peroxidase and SOD in soils of bamboo forest

	Fresh soil	Dried soil	Decrease of enzyme activities after dried (%)
Catalase activity (U g^{-1} FW)	1.581±0.16	1.286±0.12	18.7±7.6
Peroxidase activity ($\times 10^{-3}$ $\triangle OD_{470}$ $min^{-1} g^{-1}$ FW)	3.5±0.3	1.3±0.2	62.9±5.7
Superoxide dismutase (U mg^{-1} protein)	0	0	–

Soil samples were collected from three different places of the forests, each with three independent repeats. All samples were assayed at least three times.

But no SOD activity was detected in all soil samples from the bamboo forests. As an alternative control to test the method for SOD assay, SOD activity in apple fruits was analyzed and was comparable to reported results (data not shown). Drying treatment of the soil at 70℃ for 4 h resulted in a 18.7% decrease in catalase activity, and a 62.9% decrease in peroxidase activity.

3 Discussion

There are several methods for the detoxification of toxic compounds in wastewater and soils, such as biodegradation, chemical and physical degradation, and sorption or fining [13,18,26-29]. Biodegradation has become an attractive way, as it is economically and environmentally safe, and can take place under natural conditions as well. However, biodegradation of chemicals, in many cases, could be in slow rate, and was dependent on soil and environmental conditions for the growth of microorganisms [18,27]. Adsorbents or fining reagents, such as activated carbon, fibres, gels or colloids, can be used to remove a wide range of compounds, particularly phenolics and anthocyanins, from wine, wastewater and soils. But it does not mean that they can degrade or decompose toxic substances. Activated carbon has been used to detoxify allelochemicals by directly applied on soil surface or incorporated into soil [30-32]. H_2O_2 is one of the powerful oxidants for oxidation of toxic chemicals, particularly phenolic compounds. Chemical degradation with Fenton's reagent was considered to be a good alternative for degradation of environmental toxic compounds[33-37].

Our results showed that Fenton's reagent could efficiently oxidized *p*-coumaric, *p*-hydroxybenzoic acids, and juglone by 75%, 90% and 100%, respectively, in a very short period at room temperature, which were simily to the work by Mantzavinos[18] and Peres et al.[19]. However, in both cases with *p*-hydroxybenzoic and *p*-coumaric acids, the phenolics in the reaction solutions could not be completely degraded by the Fenton's regeant. Mantzavinos[18] proposed that this implied the presence in the reaction mixture of oxidation intermediates that are refractory towards the Fe^{2+}/H_2O_2 system. Direct treatment of soil with the Fenton's reagent resulted in a significant decrease (32% to 37%) of *p*-coumaric acid in the soil. However, compared with a 75% degradation of *p*-coumaric acid in both standard compounds and soil extracts, direct application of Fenton's reagent to soil had a low efficiency of degradation. Several factors might affect the degradation of phenolics in soil. Soil antioxidant enzymes, which could directly decompose H_2O_2 into CO_2 and H_2O, might be one of the important factors. Enzyme activities of catalase and peroxidase were detected except SOD in the soil beneath the bamboo forests (Table 1). These two enzymes could decompose H_2O_2 applied to the soil and therefore prevent its degradation on phenolics in soil. However, on the other hand, the two enzymes could function for clearing surplus H_2O_2 applied to soil. Tsai and Young[16] reported that soil phenolics are mainly in bound forms. Soil phenolic compounds we analyzed are total phenolics, including free and bound forms. The Fenton's reagent may not be able to react efficiently with bound forms of phenolics. Repeatedly application of H_2O_2 at different intervals or seasons

may be able to maintain a low level of phenolics in soil. Our results indicated that the Fenton's reagent was a good reagent for the degradation of toxic allelochemicals in plant soils and cultural solutions.

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