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Table: -, Figs : 7

Allelopathic effects of decaying tobacco leaves on tobacco seedlings

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ABSTRACT

To find the mechanism of soil sickness problem in tobacco continuous cropping, the effects of decaying tobacco leaves and their allelochemicals on the seedlings growth of tobacco were investigated in pot experiments in greenhouse from 2008 to 2010. Results indicated that the decaying tobacco leaves released the allelochemicals (such as benzoic acid and phthalate) and their maximal release occurred 30 to 45-days after incubation. The application of these allelochemicals decreased the activity of antioxidant enzymes (3.9-30.1%) in tobacco roots, reduced the uptake of N, P and K (10.1-37.5%) and inhibited the tobacco growth (27.1-66.3%). Furthermore, the applied allelochemicals also decreased the microbial biomass C and N (13.9-57.5%) and the concentration of available nutrients (9.9-37.2%) in the rhizosphere soil of tobacco seedlings. The magnitude of allelopathic effects followed the order: the mixture of benzoic acid and phthalate > phthalate > benzoic acid > decaying tobacco leaves > control. These results suggested that the decaying tobacco leaves produced allelopathic effects on themselves. After crop harvest, the residual tobacco leaves litter should be removed from the field.

Key words: Allelochemicals, benzoic acid, decaying tobacco leaves, phthalate

INTRODUCTION

Tobacco is major cash crop in China. Due to the decrease in cultivated soils, some tobacco seedlings were planted in continuous systems in Central China (13,30). The continuous planting of tobacco lead to serious problems in fields including poor plant growth and decline in quality and production over time (6,13,25). Although the problems of continuous tobacco barrier have been solved by rice/tobacco rotation, however, the mechanism for the release of allelochemicals from tobacco tissues was poorly understood (7). Allelochemicals are low-molecular-weight compounds released from the live or decomposed plant tissues during growth (5,9,16,18). These compounds can accumulate in the rhizosphere of plants and may be toxic to neighbouring plants or themselves (18). Asao *et al.* (1) indicated that lactic acid, benzoic acid, m-hydroxybenzoic acid, p-hydroxybenzoic acid, vanillic acid, succinic acid and adipic acid in the root exudates of taro inhibited the growth of taro. Allelopathy is the release of organic compounds by plants or microorganisms that affects themselves, other plants or microorganisms (21). Many allelochemicals are present in wheat (26), ryegrass (10), mustard (14),

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Phaeodacytylum tricornutum (28), etc. Allelochemicals in continuous tobacco cropping could produce oxidative stress and increase the membrane permeability (29), however, the effects of allelochemicals on antioxidant enzyme activities remains unclear. Wu *et al.* (26) indicated that the allelochemicals present in root exudates came from wheat roots and shoots, and shoots contained higher content of allelochemicals than roots. Jia *et al*. (13) reported that tobacco roots exude many types of allelochemicals (benzoic acid, phthalate acid, etc.), however, their source is unknown, whether tobacco leaves release these allelochemicals. Moreover, information on the allelopathic effects of tobacco leaves is not known. However, the reports that tobacco seedlings release allelochemicals are lacking (29). This study aimed to investigate the allelochemicals released from the decaying tobacco leaves and their effects on tobacco seedling growth.

MATERIALS AND METHODS

I. Pot culture

Tobacco seeds (cv. 'K326') were provided by China Tobacco Hunan Industrial Co. LTD, Changsha. The soil for pot experiments was collected from our University Campus. The physical-chemical properties of experimental soil were: pH 5.5, organic matter 18.1 g kg^{-1}, hydrolysable N 55 mg kg^{-1}, available P 20 mg kg^{-1} and ammonium acetate-extractable K 101 mg kg^{-1}, respectively. Before transplanting, 3.4 g N (Urea), 1.2 g P ($(NH_4)_2HPO_4$) and 2.6 g K (KCl) were added to 10 kg soil. The experimental treatments were : (i). Control (100 mL tap water), (ii). Decaying tobacco leaves (100 mL extract of decaying tobacco leaves), (iii). Benzoic acid (100 mL of 5 mM benzoic acid), (iv). Phthalate (100 mL of 5 mM phthalate), (v). Mixture of benzoic acid + phthalate (100 mL solution containing 5 mM benzoic acid and 5 mM phthalate). Each treatment was replicated 5 times. Each pot (height 30 cm and diameter 25 cm, contained 10 kg soil) had one tobacco seedling.. Tobacco seedlings were irrigated, when the soil moisture was less than 70% of field capacity. Experiments were done in greenhouse, temperature ranged 25 to 35 □ (highest temperature was 35 □ at noon; the lowest temperature was 25 □ at night). Due to the big size of tobacco seedlings at maturity stage, tobacco seedlings were harvested at 60-days after transplanting.

II. Leaf extracts

Dry tobacco leaves were grounded into powder. The soil was passed through 1 mm mesh. Tobacco leaf powder (250 g) was mixed with 250 g soil and 750 mL water (1:1:3 ratio). Then the mixture was incubated in an incubator at 25 □ for 40 days. High ratio of tobacco leaves added into mixture was due to lower release of benzoic acid and phthalate from decaying tobacco leaves. After incubation, the mixture was extracted with 80% methanol for 30 min in 1 : 4 ratio (w/v) (11). The extraction solution was dried by a rotary evaporator (EYELA, Japan) and the dried residual was dissolved in 1 mL deionised water and used for treatment as the decaying tobacco leaves. During the incubation of tobacco leaves with soils, photographs were taken (Fig. 1).

III. Allelochemicals

After incubation, the 25 g sample of tobacco leaves decaying in soil was extracted with 100 mL of 80% methanol solution for 30 min. The mixed solution was collected, evaporated at 40 □ into dryness and dissolved in 1 ml of deionised water and was filtered through 0.22 μm filter membrane before measurement. Allelochemicals in the solution were diluted appropriately and measured with Ion Chromatography (Dionex-120, USA). The washing solution was 20 mM NaOH. The separation column was IonpacAS112HC and the pressure was 0.3 M Pa. The temperature for separation was room temperature (25□). As per the standard substances, the retention time for the peaks of benzoic acid and phthalate was 6.63 and 9.52 min, respectively. Noteworthy, benzoic acid and phthalate were not identified from the extracts of microorganisms of decaying tobacco leaves. The results suggested that the allelochemical benzoic acid and phthalate originated from leaves, rather than from the microorganisms metabolites.

IV. Tobacco growth

Treatments were applied 7-days after transplanting of tobacco seedlings in pot soil. Every 3-days, tobacco seedlings were watered with 100 mL tap water, leaf extracts, benzoic acid, phthalate or mixture of benzoic acid and phthalate as per treatments. On other days, tobacco seedlings were watered with tap water to keep the soil moisture at 70% of field water-holding capacity. After 60-day transplanting, tobacco seedlings were harvested to determine the biometric parameters, nutrient contents and enzyme analysis. Plant height and root elongation were recorded with a ruler. The soil in the rhizosphere of tobacco seedlings was also collected to determine the available nutrients, C and N and microorganisms biomass.

V. Nutrients Analysis

After harvest, tobacco seedlings were dried and ground into powder. Tissue samples of 0.5 g were digested with H_2SO_4-H_2O_2. After appropriate dilutions, the digested solution was used for N, P, and K analysis. Soil available N, readily available P and readily available K were also measured (3). A diffusion assay was used to determine the hydrolyzed N. Briefly, soil was hydrolyzed by 1.0 M NaOH and produced ammonia was absorbed by H_3BO_3. Then it was titrated by 0.01 M H_2SO_4. The amount of hydrolyzed N was calculated according to the consumption of H_2SO_4. Available P was measured by using Bray I method. The extracting solution was composed of 0.03 M ammonium fluoride and 0.1 M HCl. Soil available K was extracted by 1 M ammonium acetate. Please see Bao (3) for the detailed description.

VI. Antioxidant enzymes activity

After harvest, tobacco roots of 0.5 g was excised and ground in a 2 mL ice-cold 50 mM phosphate solution buffer (pH 7.8). The solution was centrifuged at 4□ for 15 min at 15000 *g* and the supernatant was collected to measure the enzyme activity. To determine the superoxide dismutase (SOD) (EC1.15.1.1) activity, nitroblue tetrazolium (NBT) was used to inhibit the photochemical reduction. One unit of the activity of SOD was defined as being present in the volume of extract that caused the inhibition of the photo-reduction of NBT by 50%. Catalase (CAT) (EC1.11.1.6) activity was measured by calculating the decline in absorbance at 240 nm due to the decline of extinction of H_2O_2. The reaction

mixture was composed of 25 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 0.1 mL enzyme extracts. The reaction was started by adding hydrogen peroxide. POD activity was detected by measuring the increase in absorbance at 470 nm due to guaiacol oxidation. An increase in absorbance per minute at 470 nm was defined as one unit of the enzyme activity (19).

VII. Microbial Biomass

After harvest of tobacco seedlings, the soil samples from the rhizosphere of tobacco seedlings were collected. The soil samples were dried and then kept in a refrigerator (4□) for further use. Briefly, air-dried soil of 10 g was incubated with chloroform for 1 day under vacuum conditions. The control soil was kept under vacuum conditions without chloroform. Microbial biomass was the difference with and without chloroform treatment. Each treatment was replicated for 5 times. Microbial biomass C and N were determined by chloroform fumigation-extraction, for the details, please see Bailey et al. (2).

Statistical Analysis

The experiments were done in completely randomized design and all treatments were replicated 5-times. Each experiment was replicated twice independently. The data were analyzed statistically by Microsoft Office Excel 2000 and by Duncan's New Multiple range test, $P<0.05$. Significant difference was analysis by using SAS software (SAS 8.1).

RESULTS AND DISCUSSION

Tobacco leaf decay in soil

To determine if tobacco leaves contain allelochemicals, the dry tobacco leaves were ground into powder, and then mixed with soil and water. After a complete mixing, they were then incubated in an incubation chamber for different periods. Fig. 1 indicated the colour variation of mixtures and the growth of microorganisms at different incubation periods. After 1-day incubation, many colonies of microorganisms appeared on the surface of mixture (Fig. 1A). Noteworthy, no microorganisms were observed in the soil without tobacco leaves (data not shown). The colour of colonies became dark and the colonies became bigger with increase in incubation periods (Fig. 1B-E). Since many colonies appeared on the surface of mixture, the sample was mixed again and thoroughly after 7-d incubation. Fig. 1F showed the photo at 14-d after incubation. The dark substances became black from the surface to inside of mixture. As per reports (8,11,21), the dark substances indicate the oxidation of phenolic acid–like substances, which resulted in the formation of black substances. The results of Fig. 1 indicated that the decaying tobacco leaves might release phenolic acid-like substances. However after 14-d incubation, the dark colour of the sample changed slightly from Fig. 1F to 1I.

Allelochemicals release

Our previous study indicated that the continuous cropping of tobacco seedlings

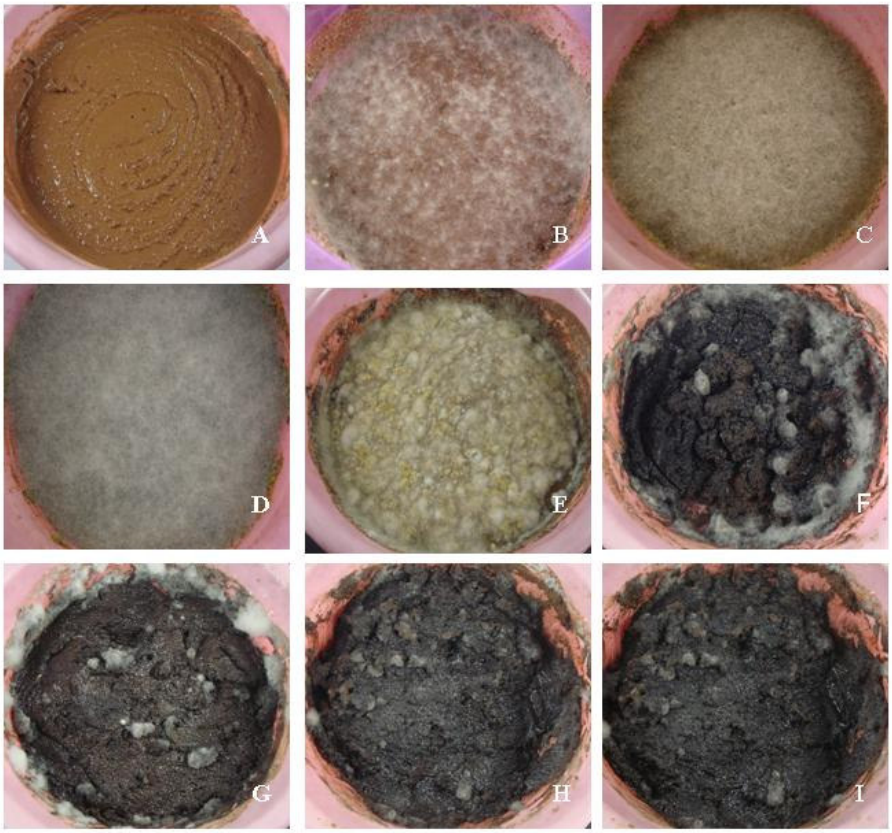

Figure 1. Tobacco leaf decaying in soil at different incubation periods. A: 0-d incubation; B: 1-d incubation; C: 2-d incubation; D: 4-d incubation; E: 7-d incubation; F: 14-d incubation; G: 21-d incubation; H: 28-d incubation; I: 35-d incubation.

could secrete some allelochemicals (such as benzoic acid, phthalate etc.), but it remained unclear from where the allelochemicals came from (13,24). To examine whether tobacco leaves contain allelopathic substances, the decaying tobacco leaves were extracted with 80% methanol solution. The benzoic acid and phthalate were identified in the extract solution. The release of both benzoic acid and phthalate increased with increasing incubation periods up to 35-days incubation (Fig. 2). The maximal release rate reached at 35-day after incubation and thereafter no increase in allelochemicals was observed (Fig. 2). The concentrations of benzoic acid and phthalate were 2.4 to 13.8 mmol kg^{-1} fresh weight of tobacco leaves. Consistent with our results, the concentration of allelochemical phenolic compounds ranged from 0.99 to 10.9 mmol kg^{-1} fresh weight in wheat shoots (26). Under natural conditions, these compounds present in plant tissues might not exhibit allelopathic potential and they may exert allelopathic effects on themselves when the tissues fall into

soils and these compounds are released. Kobayashi *et al*. (15) and Wu *et al*. (26) indicated that phenolic compounds secreted by wheat roots came from both shoots and roots of wheat seedlings. High amounts of allelochemicals in tobacco leaves might provide a possible chemical basis for the direct release of these compounds into the growth medium to exert allelopathic activity. The study of autotoxic substances in root exudates of tobacco seedlings supported this view (13).

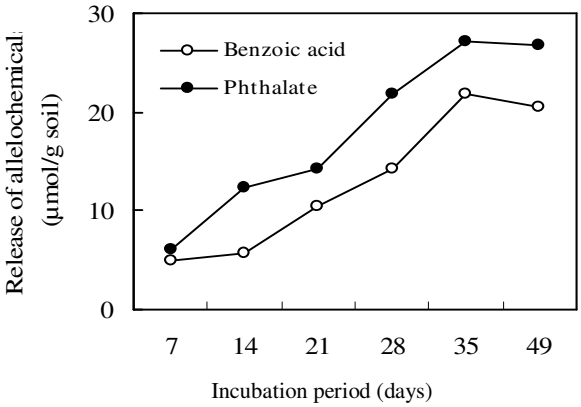

Figure 2. Dynamics of allelochemicals released from the decaying tobacco leaves. Leaf extracts were extracted at 7-, 14-, 21-, 28-, 35-, 49-days after incubation. Benzoic acid and phthalate were measured by Ion Chromatography. The data is mean of 5 replicates.

Allelochemicals and antioxidant enzymes activity

Since benzoic acid and phthalate were identified as allelochemicals in root exudates of tobacco seedlings (13), their effects on the activities of antioxidant enzymes were measured in tobacco roots (Fig. 3). In the roots of 60-day transplanted tobacco seedlings, the extracts of decaying tobacco leaves decreased the activities of SOD, POD and CAT by 7.3%, 4% and 8.9%, respectively over control. The 5 mM of benzoic acid and phthalate reduced the activities of SOD by 11.7% and 12.9%, POD by 10.6% and 13.3%, and CAT by 16.7% and 23.4%, respectively. Mixture of benzoic acid and phthalate was more inhibitory than single benzoic acid or phthalate treatment. In mixture treatment, the activity of POD, SOD and CAT decreased than control by 14.8%, 21.9% and 30%, respectively (Fig. 3). In agreement with our results, Lara-Nunez (17) indicated that allelochemical stress could cause significant oxidative damage, increase the membrane permeability (4,28) and inhibited the growth of *Lycopersicon esculentum* Mill (17). Allelochemical substance prohydrojasmon influences the activity of SOD, POD and CAT, and thereby could regulate the performance of wheat genotypes (31).

Allelochemicals and tobacco seedlings growth

Lara-Nunez *et al.* (17) indicated that allelochemical stress, caused by aqueous leachates of *Sicyos deppei*, not only produced an oxidative imbalance, but also suppressed

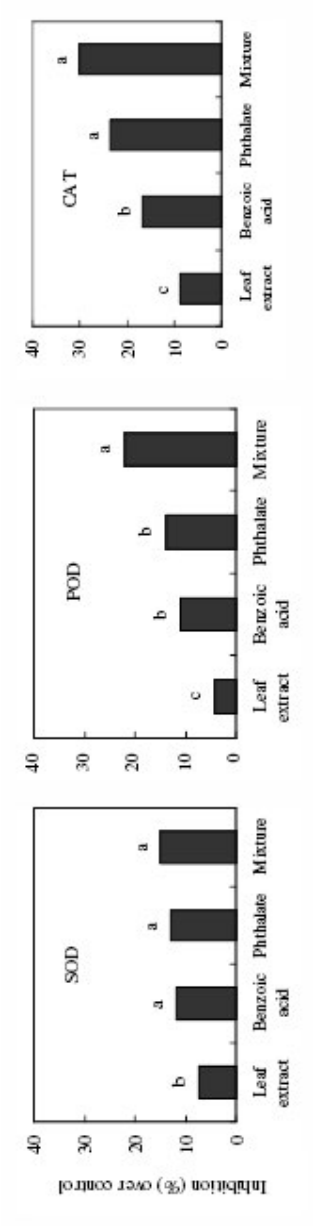

Figure 3. Inhibitory effects of allelochemicals on SOD, POD or CAT activity in tobacco roots.

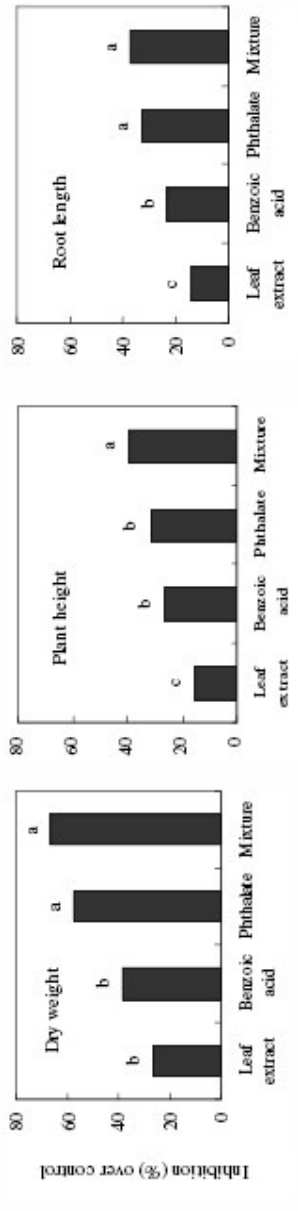

Figure 4. Inhibitory effects of allelochemicals on tobacco growth.

the plant growth. Besides the activities of antioxidant enzymes, the effects of allelochemicals on the growth of tobacco seedlings were also examined under soil cultivation conditions. The growth parameters (fresh weight, dry weight, shoot and root length) of tobacco seedlings responded similarly to different treatments (Fig. 4). The magnitude of inhibitory effects of different allelochemicals followed the order: mixture of phthalate and benzoic acid > phthalate > benzoic acid > decaying tobacco leaves > control (Fig. 4). In comparison to the control. The dry weight of tobacco seedlings was decreased by 27.1%, 38.9%, 57.5% and 66.3% under leaf extract, benzoic acid, phthalate and mixture treatment over the control, respectively. The corresponding value for root length was 13%, 23.1%, 32.3% and 36.9%.

Allelochemicals and nutrients uptake

The leaf extract or allelochemicals decreased the total N, P and K in tobacco seedlings differentially than control (Fig. 2). Total N in tobacco seedling under leaf extract, benzoic acid, phthalate, and mixture treatment was 89.2%, 80.9%, 68.4% and 53.9% of control, respectively. Similar tendencies were observed on the P and K uptake in tobacco seedling. The magnitude of allelopathic effects on nutrient uptake of tobacco seedling was: mixture of phthalate and benzoic acid > phthalate > benzoic acid > decaying tobacco leaves (Fig. 5). Phenolic compounds influences the nutrients uptake of plants (7).

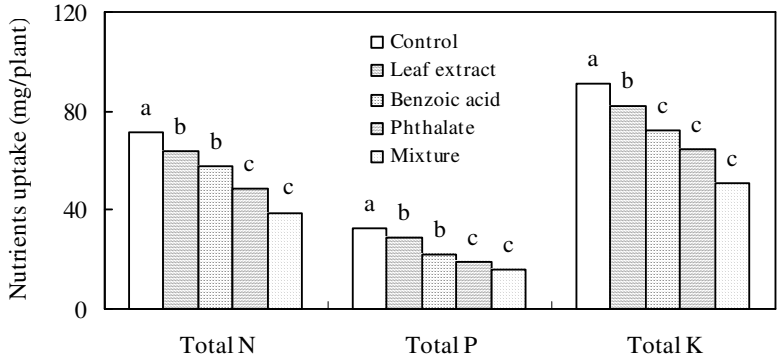

Figure 5. Effects of allelochemicals on tobacco nutrients uptake.

Allelochemicals and soil available nutrients

Xiao *et al*. (27) indicated that allelochemicals could influence the properties of soil nutrients by chelating Al, Fe, Mn etc., combining with phosphorus, or modulating the activity of P or K-solubilizing microorganisms. The allelochemicals released from the decaying tobacco leaves not only inhibited the uptake of nutrients in tobacco seedlings (Fig. 5), but also influenced the concentrations of soil available N, P and K in the rhizosphere of tobacco seedlings. The allelochemical benzoic acid, phthalate and their mixtures reduced the concentrations of soil available N, P and K significantly (Fig. 6). For example, the available K in the rhizosphere of tobacco seedling under leaf extract, benzoic acid, phthalate, and mixture was 85.1%, 79%, 69.1%, and 62.8% of control, respectively.

The concentration of soil available nutrients in the rhizosphere over the control > decaying tobacco leaves > benzoic acid > phthalate > mixture. Inderjit and Mallik (12) indicated that phenolic acid could compete with anions and chelate cations in soil solution, thus influence the release of available nutrients (22). Allelochemicals released from the decaying tobacco leaves might regulate the release of available nutrients by the above pathways. Further research work is needed to find how the allelochemicals from decaying tobacco leaves decreased the availability of nutrients.

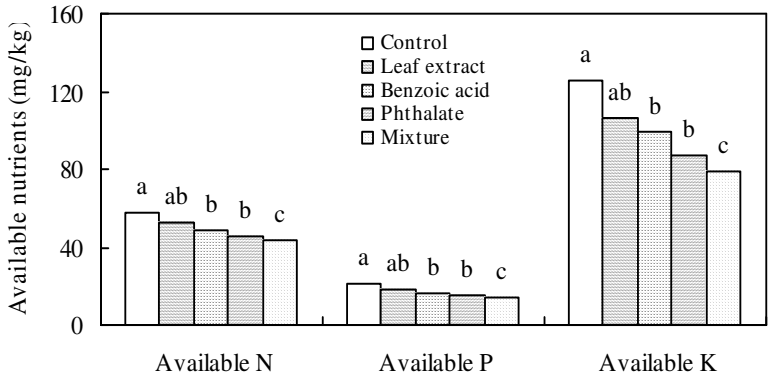

Figure 6. Effects of allelochemicals on available nutrients in the soil rhizosphere of tobacco seedlings.

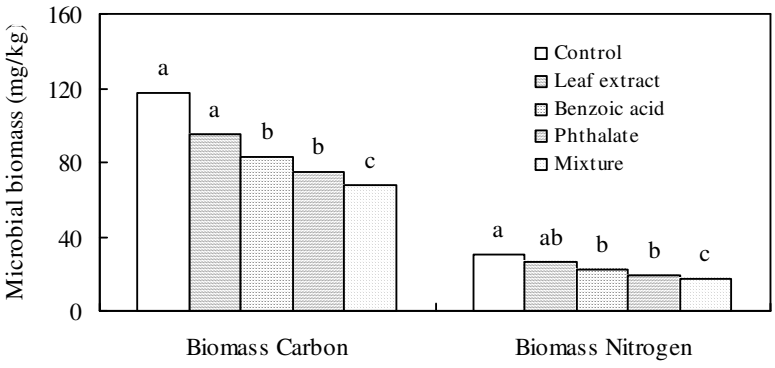

Figure 7. Effects of allelochemicals on soil microbial biomass of tobacco rhizosphere.

Allelochemicals and microorganisms

The treatment with allelochemicals decreased both microbial biomass C and N significantly (Fig. 7). However, treatment with decaying tobacco leaves influenced the microbial biomass C and N slightly. Microbial biomass C and N were in comparison to the

control 80.8% and 87.1%, 71.2% and 74.8%, 63.7% and 65.2%, 57.5% and 55.6% respectively under leaf extract, benzoic acid, phthalate, and their mixture. Microbial biomass C and N showed a similar response to different treatments (Fig. 7). The phytotoxicity of allelochemicals was generally correlated to their concentration in the soil and not their amount added to soil (11,23). Liu *et al*. (20) indicated that decomposing material of tobacco residues had allelopathic effects on the seed germination of lettuce at or above 0.10 g/mL. The phytotoxicity of benzoic acid and phthalate on tobacco growth and rhizosphere microorganisms suggested higher concentrations existed in the rhizosphere soils (Figs. 5-7). In our experiments, the level for allelopathic activity of benzoic acid or phthalate was over 0.1 g/g soil, thus producing obvious allelopathic effects on the growth of tobacco seedlings (Figs. 2, 3). Kaur *et al*. (14) found that benzoic acid application resulted in disorganized, distorted and irregular shaped cells and disruption of cell organelles of mustard roots. More research work is needed to examine how benzoic acid or phthalate inhibits the cell growth of tobacco roots and activity of microorganisms in the rhizosphere of tobacco seedlings in near future.

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