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Effects of allelochemicals from tobacco root exudates on seed germination and seedling growth of tobacco

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ABSTRACT

The composition of root exudates from two varieties (K 326 and NC 89) of tobacco were identified by GC-MS. Organic acids,amides and esters were the main constituents of the root exudates which differed in their qualitative and quantitative composition.Contents of cinnamic acid and nine other organic compounds was reduced in about 29% after 15 days of exposition to soil microflora.The effects of 6 organic acids (benzoic, cinnamic, lauric, myristic, palmitic and phthalic acids) identified intothe root exudates of tobacco varieties were evaluated on seed germination of tobacco.The cinnamic and benzoic acids were the most inhibitory on seed germination.

Key Words: Allelochemicals, allelopathic effects, continuous cropping, GC-MS, germination, organic acids, root exudates, seeds, tobacco.

INTRODUCTION

Autotoxicity due to continuous cropping reduces soil quality and both the yield and quality of tobacco leaves in China (7,12,13,19). In some zones, this problem has been solved by rice/tobacco rotation (9). Althougth the reasons involved in tobacco autotoxicity are still unknown, root exudates of the tobacco plants seem to have major role.Root exudates might include allelochemicals which can (i).affect the growth of tobacco plants, (ii).inhibit the growth of useful soil bacteria, (iii). promote the reproduction of harmful bacteria and (iv).destroy the balance of rhizosphere microecology, which results in more pathogens and soil-borne diseases (11).The allelochemicals present in the root exudates of tobacco showed to inhibitseed germination of tobacco and lettuce (10,15).Fractions of the root exudates obtained in acidic, neutral and alkaline conditions also inhibits seed germination of tobacco (3).However, the chemicals involved in this inhibitory activity are not fully known.This study aimed to identify the organic chemicals into the tobacco exudates and their effects on the seed germination of tobacco.

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MATERIALS AND METHODS

The experiments were conducted from May to October of 2011 at the Tobacco Resources and Environment Field Station, Chinese Academy of Agricultural Sciences, Qingdao, (Latitude : 36°38′, longitude : 120°45′, altitude : 0 m). The varieties of tobacco 'K 326' and 'NC 89' were obtained from Shandong Tobacco Seed Company. The benzoic , cinnamic, lauric, myristic, palmitic and phthalic acids were obtained from Dr. Ehrenstorfer company, Germany.

The seedlings of both varieties were grown in pots (50 cm dia, height: 30 cm) filled with acid washed quartz sand for 40 days in order to identify the organic chemicals in the root exudates. The pots were watered daily with 200 ml of a nutrient solution composed of (g/L): $Ca(NO_{3)2}$, 1.90; $NH_4H_2PO_4$, 0.24; $Mg(SO_4)H_2O$, 0.98; K_2SO_4, 1.029; $CaCl_2$, 0.894; KNO_3, 1.22; H_3BO_3, 1.90; $MnCl_2{\cdot}4H_2O$, 1.8; $ZnSO_4{\cdot}H_2O$, 0.22; $CuSO_4{\cdot}5H_2O$, 0.08; $H_2MoO_4{\cdot}4H_2O$, 0.09; EDTA-Fe, 0.05.

Extraction and collection of tobacco root exudates

Tobacco seedlings were cultivated for 40 days in nutrient solution, then transplanted in 10cm x 10cm x 35cm containers (one seedling /per container) and irrigated with 500 ml deionized water, after 24 h,the water was replaced with fresh deionized water. The culture liquid collected for 2 days was processed further as the root exudates.To identify the organic compounds in root exudates, the culture liquid were extracted with neutral ethyl acetate. Following a modified protocol (14), 500 ml root exudates were mixed with 250 ml neutral ethyl acetate and kept still for 5 min. Then root exudates liquid was extracted under neutral conditions, the remaining liquid was adjusted to pH 2.0 with 1 mol/L HCl, then mixed with 250 ml ethyl acetate and kept still for 5 min again, then root exudates liquids were extracted under acidic conditions, the remaining liquid was adjusted to pH 8.0 with 1 mol/L NaOH, then mixed with 250 ml ethyl acetate and kept still again for 5 min Then root exudates liquids were extracted under alkaline conditions, as per the flow diagram (Fig. 1). The extracted liquids were vacuum concentrated to 1ml (at 45^{o}C) and filtered with 0.45µm membrane .

Silylation of root exudates

One ml concentrated extraction solution was put into a 2 ml glass tube and added 0.25 ml of silylation reagent (NA: BSTFA:TMC= 5:1). The tube was sealed and placed in water bath at 80℃ for 2 h (2).A Volume of 1µl of the silylated products was injected into a GC–MS equipped with a capillary HP-5MS column (30m×0.25mm×0.25µm), with helium as the carrier gas at a flow speed of 1 ml/min, an inlet temperature of 250°C.The MS functioned with an electron bombardment voltage of 70eV, ion source temperature of 230°C and scan range M/Z 30-600AMU.The GC program started with 50°C for 2 min,then increased to 250°C at the rate of 6ºC/min,then kept for 15min. Identity of eluted compounds was determined by comparing their mass spectra with NIST98 mass spectrometry database.

Decomposition of root exudates

Fresh soil collected from the pots containing the tobacco plants was sieved to 2

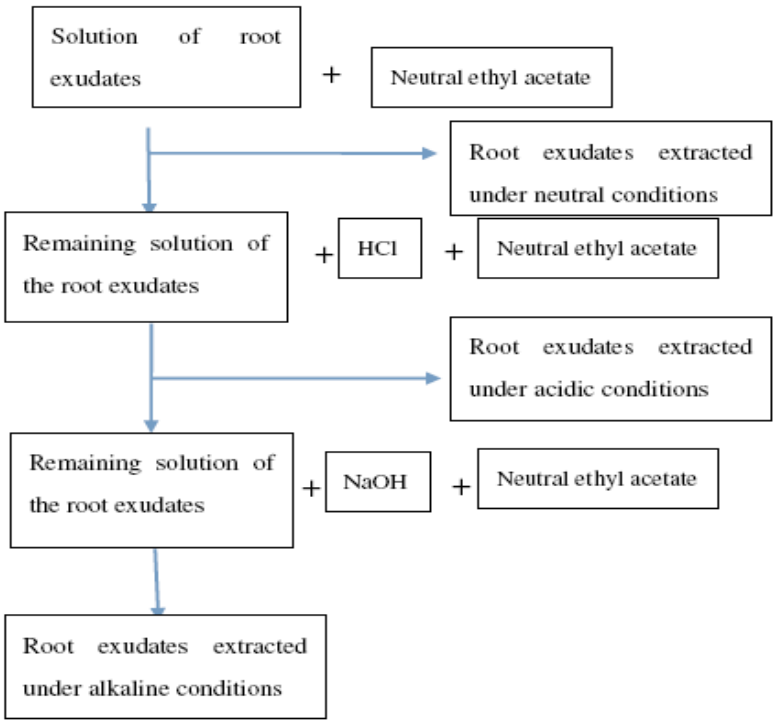

Figure 1. Flow diagram of root exudates extraction procedure

mm and adjusted to 60% of water holding capacity. Then, 500 g of this soil was kept at 25^0C in the darkness for 15 days, placed in a small cup and exposed to a NaOH solution (1mol • L^{-1}) to absorb CO_2 released by soil respiration.The soil in the cup was sampled once every 3d. Each sample consisted in25 g of wet soil which was added to a 50 ml centrifuge tube containing 25 ml of NaOH (1mol • L^{-1}), After stilling for 12 h, the sample was stirred at 180 rpm • min^{-1} for 30 min, and then centrifuged for 7 min (6000 rpm • min^{-1}).The pH of the supernatant was adjusted to pH 2.5 with HCl (12 mol • L^{-1}), and centrifuged (6000 rpm • min^{-1}, 7 min) for the remotion of humic acid. Then the supernatant was passed through a 0.22μm cellulose film. The filtrated liquid was injected (10 μl) in an Agilent 1290-6430 ultra-HPLC coupled to a triple quadrupole Tandem Mass Spectrometer. Nicotine was detected by positive ion mode and the other substances by negative ion detection mode. The apparatus was equipped with an ACQUITY UPLC®HSS T31.8μm 2.1×150mm Column. The mobile phase was 5% acetonitrile in H_2O (solvent A) and 100% acetonitrile (solvent B) provided at a flow speed of 0.2 ml/min. The gradient program was 0% to 25% B (15 min), 25% to 80% B (5 min), 80% to 0% B (1 min), and 0% B (9 min). Column temperature was 25°C. The MS operated with ESI as ion source at an atomization gas pressure of 40 psi, a drying gas temperature of 350°C, a drying gas flow speed of 10L/min, the capillary voltage in positive ion mode (4kV) and in negative ion mode (-4KV), with a scan range of 50-500 m/z.

Allelopathic effects of organic acids on seed germination of tobacco

The effects of organic acids identified in the root exudates of the 2 tobacco varieties (K 326 and NC 89) were studied on the seed germination of the tobacco variety 'ZhongYan 104'. Fifty seeds were disinfected with 0.5 mol·L^{-1} $CuSO_4$ for 10 min, washed with tap water, dried and then placed in a Petri plate of 10 cm in diameter. Five ml of a solution of an organic acid was added in each Petri plate. Concentrations assayed were 0.1g/L,0.25 g/L and 0.5 g/L. Five ml of water was added in each Petri dish used as control instead of the solutions of organic acids. The seeds were kept on two layers of filter papers, and incubated at 28^{o}C under a light of 4000 Lux for 7 days. Each test was repeated three times. After 7 days, the root length and dry weight of 20 seedlings were determined. Germination percentage was calculated by counting the number of germinated seeds out of 50.

Statistical Analysis of Data: Data were subjected to one way ANOVA and the significance of means was determined by Tukey's pair-wise multiple comparison tests (α = 0.05) test, using the software SAS 9.1.3.

RESULTS AND DISCUSSION

Identification of allelochemicals by GC-MS

Root exudates can be phytotoxic on receptor plants by several mechanisms including changes in pH and osmotic potential of the soil surrounding the receptor plants (5), Hence, the presence of allelochemicals in the neutral, acidic and alkaline extracts obtained from root exudates of the tobacco varieties were investigated in order to confirm their allelopathic activity. The GC-MS analysis of the neutral, acidic and alkaline extracts from the root exudates indicated that some allelochemicals were released by both varieties while other compounds were unique of one of them. The neutral extract of K326 contained 19 compounds while NC89 had 29 compounds. Both varieties showed the presence of benzoic acids, 4-hydroxybutyric acid, succinic acid, palmitic acid, hexadecenoic amide, cyclopentane, stearic acid amide and scopoletin (Table 1). In the case of the acidic extracts, K326 and NC89 contained 42 and 47 compounds, respectively, while **t**he common compounds were 4-hydroxybutyric acid, benzoic acid, succinic acid, palmitic acid, cyclopentane, scopoletin, hexadecenoic amide and stearic acid amide (Table 2). In the alkaline extracts, **t**he K 326 included 29compounds and NC 89 contained only 14 compounds. The common organic components were 2-hydroxy propionic acid, 3-methyl-2-hydroxy acid, 4-hydroxybutyric acid, benzoic acid, succinate, phenylpropionate, cinnamic acid, lauric acid, 3,4-dihydroxybenzoic acid, myristate acid, palmitic acid, oleic acid, stearic acid, dibutyl phthalate, diotyl adipate, cyclopentane, ring tetradecane and nicotine (Table 3). The release of phthalates and alkanes was previously reported in root exudates of tobacco variety G168 under continuous cropping (6). In our work, however, it was noticeable that most of the molecules identified in the root exudates were organic acids and the esters were the molecules found in the highest concentrations. The intraspecific variability detected seems to confirm variations in the activity of root exudates among tobacco varieties due to qualitative and quantitative changes in the release of allelochemicals.

Table 1. The composition (%) of tobacco root exudates extracted under neutral condition (pH 7.0)

Organic compound	Root exudates in Tobacco varieties	
	Variety K326	Variety NC89
2-methyl-propionic acid	0.31	-
Methoxy-oxalic acid	1.53	-
Cinnamic acid	0.28	-
Myristic acid	0.26	-
2-hydroxy propionic acid	-	0.58
Octanoic acid	-	0.04
Azelaic acid	-	0.18
Phenylpropionic acid	-	0.06
Capric acid	-	0.14
3-cinnamic acid	-	0.50
Lauric acid	-	0.19
3-methyl-4-hydroxybenzoic acid	-	0.08
Myristic acid	-	0.43
Palmitic acid	-	0.33
Oleic acid	-	1.73
Stearic acid	-	1.64
4-hydroxybutyric acid	0.40	0.11
Benzoic acid	0.14	0.10
Succinic acid	3.00	0.58
Palmitic acid	1.40	1,78
Total organic acids	**7.44**	**8.47**
Cyclopentane	0.05	0.36
Ring tetradecane	0.28	-
Nonadecane	0.53	-
Docosane	-	0.56
Total alkanes	0.86	0.92
Acetic acid-2 phenyl ethyl	0.54	-
Dibutyl phthalate	48.14	-
Scopoletin	0.77	3.02
Dioctyl Adipate	19.12	-
Dioctyl phthalate	0.52	-
Diethyl phthalate	-	31.17
Glycol diethyl octyl	-	13.07
Total Esters	**68.55**	**47.26**
Hexadecenoic amide	1.15	1.18
9-18-ene amide	7.14	-
Stearic acid amide	0.69	7.3
Total amides	**8.98**	**8.48**
4-Hydroxy-3-methoxyacetophenone	0.52	-
Oleic acid nitrile	0.89	-
Glycerine	-	0.15
Nicotine	-	0.49
3-methyl-4-hydroxybenzaldehyde	-	0.11
Oleic acid nitrile	-	1.29
4-hydroxy-3-methoxy-acetophenone	-	0.91
Total root exudates components	**87.24**	**71.1**

Table 2. The composition (%) of tobacco varieties root exudates extracted under acidic condition (pH 2.0)

Organic compound	Root exudates in Tobacco varieties	
	Variety K326	Variety NC89
2-hydroxy propionic acid	1.20	3.77
3-hydroxybutyric acid	1.00	-
3-methyl-2-hydroxy acid	0.33	0.17
4-hydroxybutyric acid	1.14	1.47
2-hydroxy-hexanoic acid	0.45	-
Benzoic acid	0.45	0.45
Succinate acid	1.28	1.31
Phenylpropionate	0.13	0.13
Cinnamic acid	0.55	1.05
3-hydroxybenzoic acid	0.08	-
4-hydroxyphenylacetic acid	0.25	-
Lauric acid	0.09	0.12
3-hydroxy-4-methoxy-benzoic acid	0.27	-
3-methoxy-4-hydroxyphenyl acetic acid	0.26	0.12
Azelaic acid	0.92	-
3,4-dihydroxybenzoic acid	0.36	0.76
Myristate acid	0.46	0.22
4-methoxy-3-hydroxy cinnamic acid	0.19	-
Pentadecanoic acid	0.47	-
Cis-9-hexadecyl (carbon) acid	0.20	-
Palmitic acid	12.84	1.58
3-methoxy-4-hydroxy cinnamic acid	0.35	-
Heptadecanoic acid	1.21	-
9,12-octadecadienoic acid	0.28	-
Oleic acid	0.91	0.87
Stearic acid	1.46	1.04
Dehydroabietic acid	0.15	-
2-furoic acid	-	0.08
Hydroxybutyrate	-	0.79
2-hydroxy-4-methyl valerate	-	0.41
Nicotinic acid	-	0.06
Phenylacetic acid	-	0.05
6-hydroxy-3- pyridinecarboxylic acid	-	0.08
3-hydroxybenzoic acid	-	0.12
4-hydroxyphenylacetic acid	-	0.21
Phthalic acid	-	0.12
Azelaic acid	-	1.09
3-methoxy-4-hydroxyphenyl propionic acid	-	0.04
4-methoxy-3- hydroxy-cinnamic acid	-	0.19
3-hydroxy-3- (4'-hydroxy-3'-methoxyphenyl) propionic acid	-	0.42
4-methoxy-3-hydroxy cinnamic acid	-	0.82
9,12-octadecadienoic acid	-	0.28
Dehydroabietic acid	-	0.05
Total organic acids	**27.28**	**17.87**
3-chloro-benzoic acid, 2-phenylethyl ester	0.54	-

Dibutyl phthalate	32.79	34.73
2,4-dihydroxybenzoate	0.32	
Dioctyl adipate	13.41	14.96
Scopoletin	0.44	0.3
Acetic acid-2- phenyl ethyl	-	0.57
Dioctyl phthalate	-	0.14
Phthalate single-(2-ethylhexyl alcohol) esters	-	8.64
Total Esters	**47. 5**	**59.34**
N-4-methoxy-phenyl-2-hydroxy amide	0.14	
Hexadecenoic amide	1.14	
9-18-ene amide	5.48	
Nutmeg amide	-	0.9
9-oleamide	-	3.36
Stearic acid amide	-	0.48
Total amides	**6.76**	**4.74**
Cyclopentane	0.21	0.09
2,2,4-3 hydroxyphenyl propane	0.6	-
Ring tetradecane	0.26	0.12
Tricosane	0.16	-
2,2-bis(4-hydroxy)phenyl propane	-	0.46
Heptadecene	-	0.16
Total alkanes	**1.23**	**0.83**
Nicotine	0.36	0.12
4-hydroxy-3- methoxyacetophenone	0.08	-
Oleic acid nitrile	0.81	0.65
Glycerine	-	0.17
Total root exudates components	**84.27**	**83.72**

Table 3. The composition (%) of tobacco root exudates extracted under alkaline condition (pH 8.0)

Organic compound	Root exudates in Tobacco varieties	
	Variety K326	Variety NC89
2-hydroxy propionic acid	0.33	0.39
3-hydroxybutyric acid	0.04	
4-hydroxybutyric acid	0.07	
Succinate acid	0.46	
Lauric acid	0.04	0.19
Myristate acid	0.16	0.29
Pentadecanoic acid	0.19	
Cis-9-hexadecyl (carbon) acid	0.4	
Palmitic acid	0.73	1.73
Oleic acid	0.27	
Stearic acid	0.93	1.05
Total organic acids	**3.62**	**3.65**
Acetic acid-2-phenyl ethyl	0.35	
Phthalate G ester	0.35	
Dibutyl phthalate	31.25	51.13
Diethyl phthalate	0.23	
Scopoletin	0.3	
Dioctyl adipate	14.41	19.49

Phthalate single-ethylhexyl ester	0.28	
Phthalate single-(2-ethylhexyl alcohol) esters		3.48
Total Esters	**47.17**	**74.3**
Dodecanamide		1.4
Stearic acid amide		8.02
Trifluoroacetamide	31.48	
Sixteen amide	0.5	
9-oleamide	2.5	
Stearic acid amide	0.6	
Total amides	**35.08**	**9.42**
2,2-bis(4-hydroxypheny) propane		0.9
Butylated hydroxytoluene	0.05	
Hexadecane	0.03	
Cyclopentane	0.1	0.23
Total alkanes	0.18	1.13
Glycerine	0.06	0.63
Nicotine	0.74	
Oleic acid nitrile	0.64	
Total root exudates components	**87.49**	**89.93**

Decomposition of the identified compounds in soil conditions

Root exudates of plants always are a complex mix of allelochemicals and non-toxic compounds. After release, the persistence of some compounds in the mix will be very short because they are more readily available for microbial utilization, and thus less likely to fuction as allelochemicals in natural conditions. This situation led us to evaluate the changes in the contents of the identified compounds in the soil recovered from the pots where the tobacco varieties were grown (Table 4). Glycerine was the less persistent compound and disappeared after 15 days. In the same period of time, about 70% of the content of four types of organic compounds (palmitic acid, stearic acid amide, dibutyl phthalate, nicotine) was degradated. The decomposition of 10-types of organic compounds (scopoletin, 4-hydroxy butyric acid, hydroxybutyrate acid, 3-methyl-2-hydroxy acid, 3,4-dihydroxy benzoic acid, lauric acid, 9,12-octadecadienoic acid, dioctyl adipate, palmitic acid, benzoic acid) reached 50% to 69%, Cinnamic acid and 9 other organic compounds (4-hydroxy phenylacetic acid, phthalate, 3-hydroxy benzoic acid, myristate, succinate,phenylpropionate acid, dioctyl phthalate, 3-methoxy-4-hydroxy phenyl acetic acid, cinnamic acid) suffered degradations equal or lower to 29% of the original contents detected. These results suggest that, based on their persistence, the last group of compounds is more likely to exert some kind of allelopathic activity. Nevertheless, several factors, including the interactions among these compounds were not investigated and further research is needed in this regard.Previous reports indicate that the esters, alkanes and amides are not allelopathic and that the organic acids are the most allelopathic to peppers, tomatoes and rice (8,15,16,19,21). For this reason, we selected 6 organic acids (benzoic acid, cinnamic acid, lauric acid, myristic acid, palmitic acid and phthalic acid) to examine their effects on seed germination of tobacco. The organic acids assayed have been reported previously as allelopathic compounds (1,4,18,21) .

Table 4. Biodegradation (%) of the organic compounds into the soil

Organic compounds	Degradation (%) Days after Incubation				
	3d	6d	9d	12d	15d
3-methyl-2-hydroxy acid	9.1	19.0	43.59	51.1	62.3
Succinate acid	4.2	21.6	28.6	34.5	36.2
Cinnamic acid	6.1	15.0	18.0	22.9	24.6
Lauric acid	8.4	28.5	38.1	53.5	57.6
Phthalate acid	7.3	20.6	28.2	43.6	47.2
3,4-dihydroxy benzoic acid	8.6	26.6	49.0	53.6	60.7
Palmitic acid	9.9	17.8	32.5	50.1	54.1
Oleic acid	14.0	33.5	61.0	74.9	79.5
Scopoletin	19.1	27.4	47.0	51.5	67.7
3-hydroxy benzoic acid	4.1	18.6	27.9	35.9	42.4
Dioctyl phthalate	5.0	17.8	26.9	33.0	36.1
Glycerine	30.0	46.1	70.3	80.8	100
4-hydroxy butyric acid	8.7	28.0	44.2	56.1	64.9
4-hydroxy phenylacetic acid	8.1	15.8	35.4	41.5	48.1
Benzoic acid	8.6	28.6	42.4	48.0	53.5
3-methoxy-4-hydroxy phenyl acetic acid	7.0	17.3	19.6	22.8	29.8
Hydroxybutyrate acid	11.0	19.8	27.3	50.8	64.7
Myristate acid	5.6	21.4	41.2	55.5	41.5
Phenylpropionate acid	8.6	16.2	30.4	37.0	41.2
9,12-octadecadienoic acid	7.3	39.2	48.0	55.8	57.8
Stearic acid amide	15.3	44.9	58.9	68.4	73.5
Dioctyl adipate	13.2	40.4	53.3	53.2	54.7
Dibutyl phthalate	13.2	19.1	34.0	63.7	71.5
Nicotine	20.6	38.9	50.5	60.3	71.4

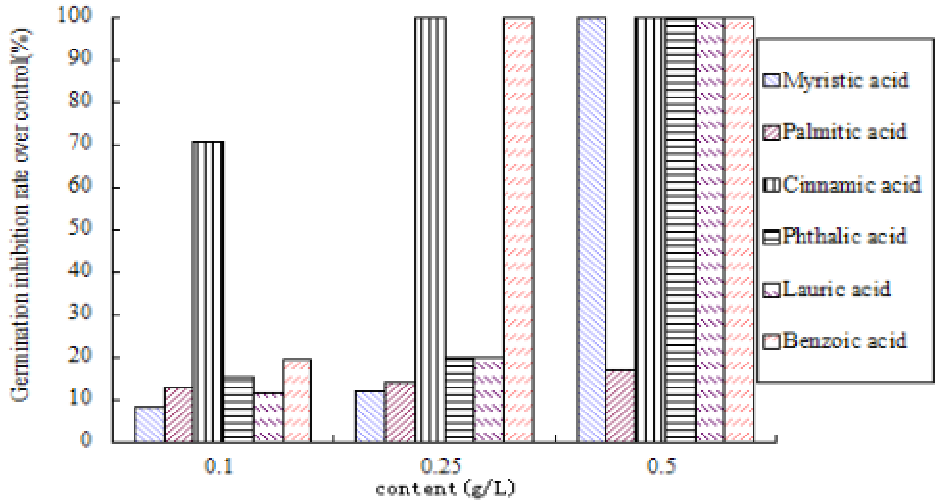

Figure 2. Inhibitory effects of different concentrations of organic acids on germination (%) over control

Seed germination and seedlings growth

Cinnamic and benzoic acids strongly inhibited the seed germination of tobacco while palmitic acid was less inhibitory. Although there were differences in inhibition rates, all tobacco seeds germinated at concentrations of 0.1g/L of the 6 organic acids assayed. At 0.25g/L, the cinnamic and benzoic acids completely inhibited seed germination. At 0.5g/L, palmitic acid was the least inhibitory (17.1%) to seed germination (Fig. 2).

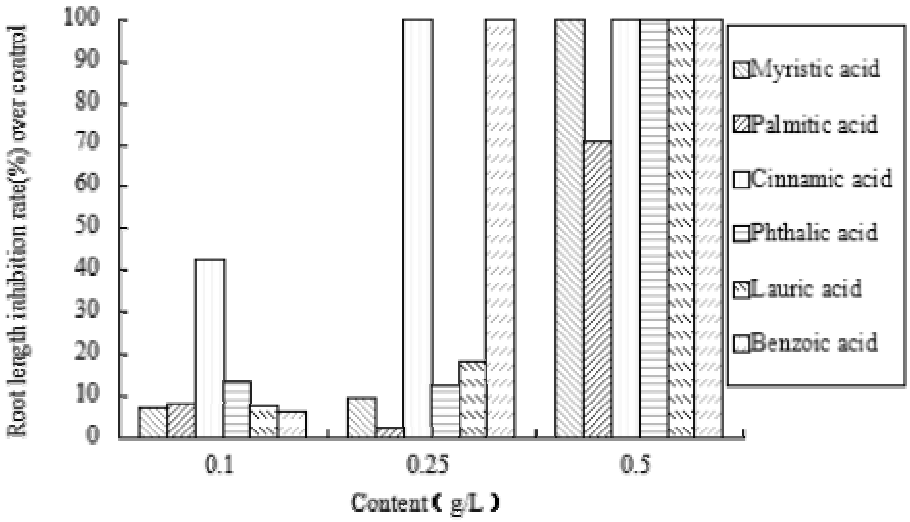

Figure 3. Inhibitory effects of various concentrations of organic acids on the root length over control

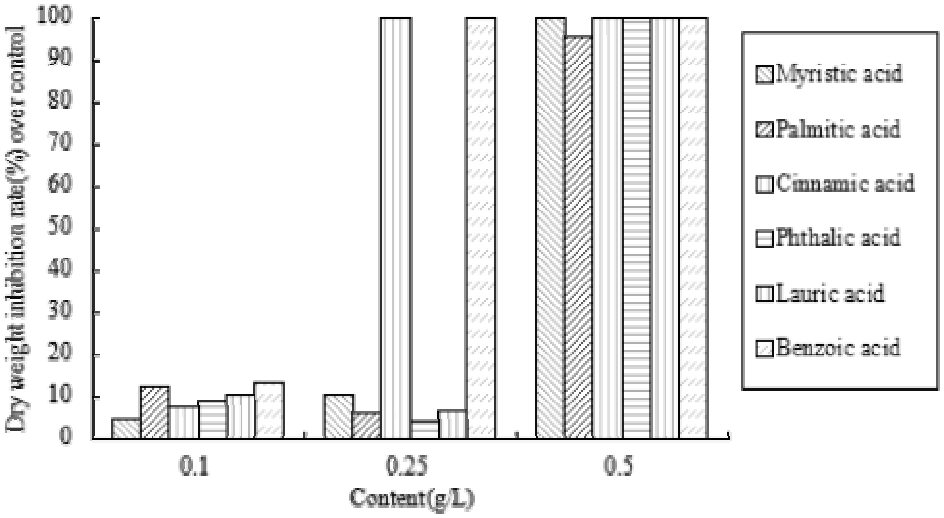

Figure 4. Inhibitory effects of different concentrations of organic acids on dry weight over control

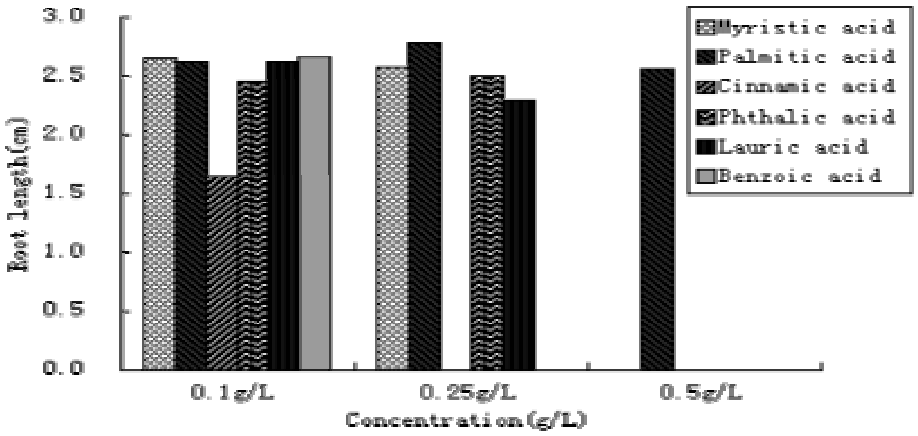

Figure 5. Root length of seedlings treated by different concentrations of organic acids

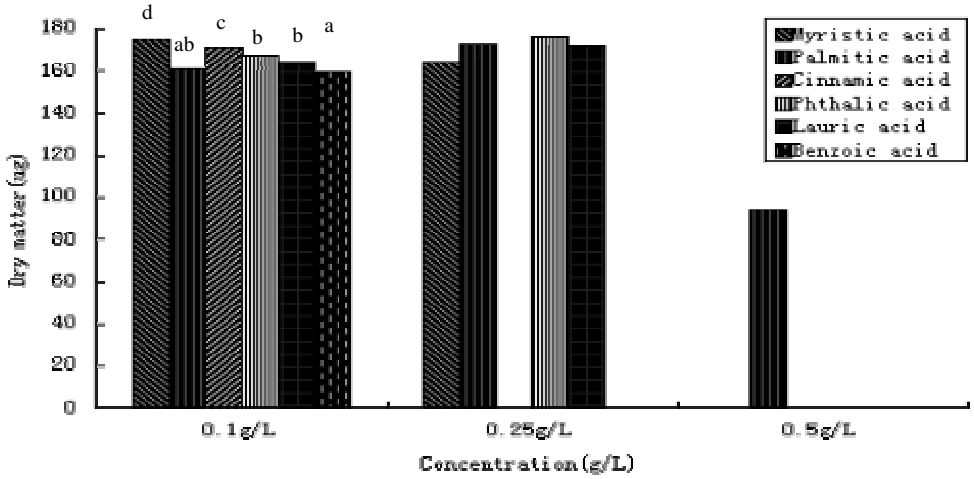

Figure 6. Root dry matter of seedlings treated by different concentrations of organic acids

The inhibition in root length and dry matter (Figs. 3 and 4) followed a similar inhibitory trend than germination. In the water control, the root length and dry matter were 2.84 cm and 184 μg, respectively. All the 6 organic acids decreased the root length and dry matter of tobacco seedlings. The cinnamic acid at 0.1g/L drastically reduced the root length to 1.64cm (Fig. 5), which was significantly lower than other concentrations ($P < 0.5$). The concentrations of these acids also influenced the dry matter of seedlings. The benzoic acid at 0.1g/L caused a maximum reduction in root dry matter (159.3μg) of seedlings (Figure 6). These results indicated that benzoic and cinnamic acids were the most allelopathic to tobacco seed germination and seedling growth.

CONCLUSIONS

Organic acids, amides and esters were the main compounds secreted by the roots of the tobacco plants. They were common components in the exudates of both tobacco varieties, but their qualitative and quantitative participation were not identical. The organic acids and the esters were in high contents. Cinnamic and benzoic acids were the most inhibitory on seed germination and seedling growth of tobacco.

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