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INFLUENCE OF ROOT EXUDATES AND RESIDUES ON SOIL MICROECOLOGICAL ENVIRONMENT

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Abstract

The effects of grape root exudates and residues on rhizosphere available nutrient, enzyme activity, microorganism quantity and population structure were determined with potted seedlings of Beta (*V. vulpine* x *V. labrusca*) as materials. Plant growth was suppressed and available P and K contents in rhizosphere soil decreased, while alkaline hydrolysis N content increased under high concentration of root exudates. As the addition ratio of root residues increased in soil, the contents of available N, P and K in rhizosphere soil increased. The correlation was found between alkaline hydrolysis N content and urease activity under the treatment of root exudates and residues, but the activity of invertase was weakened and had no obvious correlation with soil nutrient or plant growth. Polyphenol oxidase activity was also weakened when the concentration of root exudates and residues increased. The rhizosphere soil was converted from 'bacteria type' to 'fungi type' under root exudates of high concentration. Under the treatment of root residues, both bacteria/fungi and actinomycetes/fungi ratio increased in rhizosphere soil, however, fungi population diversity reduced. The beneficial *trichoderma* disappeared, while it appeared with *pythium*, *thielaviopsis* and *Stilbellales*. The results showed that the change of available N, P and K content was not the major reason that led to the depression of plant growth, while the alternation of polyphenol oxidase activity and the change of microorganism quantity and population structure might be the important reason of grape replant disease.

Introduction

The grape replant problem is expressed as stunted growth, low productivity and a decline in tree vigor leading to shorten economic life. The problem is common in all grape growing regions. The theories to explain replant obstacle on other plants include an imbalance in the physicochemical properties of soil, the allelopathy effects of root exudates and residues, and the build-up of pests and pathogens (Ruan *et al.,* 1999; Yu & Du, 2000; Noumi & Chaieb, 2011; Khan *et al.,* 2012).

Previous studies on grape replanting focused on the biotic factors (Waschkies *et al.,* 1994; Westphal *et al.,* 2002) and few studies investigated the abiotic factors. A negative effect of autotoxicity conferred by old grape roots was found in one study (Brinker & Creasy, 1998). Extensive studies of autotoxicity substance in grape root exudates and residues have led to the identification of a range of phenolic compounds including ρ-hydroxybenzoic, salicylic acid, coumaric acid, phenylpropionic acid and benzoic acid (Li, 2010). However, the cause of replant problems is complex, and it cannot be attributed to only one factor, and the interaction between different factors seems to be the major reason. (Rutto & Mizutani, 2006).

Therefore, the replant disease should be considered from rhizosphere micro-ecosystem standpoint (Ruan *et al.,* 1999). Our study investigated the effects of root exudates and residues on available nutrient content, enzyme activities and microorganism number and population structure of rhizosphere soil, aiming to reveal the reason of grape replant disease from the perspective of rhizosphere micro-ecosystem.

Materials and Methods

Plant material: This study was conducted in the vineyard of Shenyang Agriculture University, Liaoning province, People's Republic of China, from May to July in 2009. Plant materials were Beta cuttings seedling and Red Globe seedling grafted on Beta, and the seedlings grew under rain shelter.

Root exudates treatment: 2-year-old grafted seedlings of Red Globe were carefully removed from the soil and the roots were carefully washed with distilled water repeatedly. Two seedlings were put into one keg wrapped by black plastic bag, which contained 1 L Hoagland nutrient solution. The nutrient solution was aerated for 15 min every 30 min by air pump. The root exudates were collected every 2 days, and then all exudates were mixed.

One-year-old cutting seedlings of Beta, planted in circular-section pots (20 cm diameter, 15 cm height) under rain shelter cultivation were used as the testing materials. Pots received same amounts of virgin dry soil (1.3 kg), and the mean weight of pots after draining reached the amount of water corresponding to 100% of soil water holding capacity. Throughout the culture, pots were buried in soil in May, with daily water adjustments to 80% of soil water holding capacity. When the height of seedlings reach at least 5 cm long, they were treated with 300mL root exudates of different concentrations every 2 days, with 10 replicates per treatment. The treatments of root exudates were: 300mL nutrient solutions (control), 100mL root exudates + 200mL nutrient solutions (E100), 200mL root exudates + 100mL nutrient solutions (E200), 300mL root exudates (E300). Soil nutrient content, enzyme activity and microorganism number were measured on July 3rd, and at the same time all seedlings were harvested to determine plant height, stem diameter, fresh weight of shoot and fresh weight of root.

Root residues treatment: The pieces of Beta dried root were mixed with virgin soil according to the following 4 treatments, F1%: 100g soil containing 1 g dried root pieces,

F3%: 100g soil containing 3 g dried root pieces, F5%: 100g soil containing 5g dried root pieces, Control: 100g soil. Beta cutting seedlings were used as the testing materials. Cutting seedlings were transplanted into pots filled with 2 kg soil and root piece mixtures in May. Plants were cultured under shelter cultivation. Soil nutrition content, enzyme activity and microorganism number were measured on June 23rd, and at the same time all seedlings were harvested to determine plant height, stem diameter, fresh weight of shoot and fresh weight of root.

Rhizosphere soil collection: Seedlings were removed from the pots. Soil blocks were shaken off and only the soil adhering to roots was considered as rhizosphere soil (Nazih *et al*., 2001). The rhizosphere soil was collected from 10 plants for each treatment.

Soil available nutrients measurement: Alkali solution nitrogen was measured by Alkali solution-diffusion method, and available phosphorus was measured by Molybdenum blue colorimetric method. Rapidly available potassium was measured by Flame photometric method.

Measurement of urease, invertase and polyphenol oxidase: Urease was measured by indophenol-blue colorimetric method (Guan, 1986). Polyphenol oxidase was measured by pyrogallol colorimetric method (Guan, 1986) with little modifications: 1g dried soil sample that had been through 1mm sieve was put in 50mL volumetric flask. Then 10mL 1% pyrogallol was added into the volumetric flask. The volumetric flask was oscillated at 150 r·min^{-1} for 1 min and then cultured at 30° for 3h. After that 4mL citric--phosphoric acid buffer (pH 4.5) was added into the flask. Then diethyl ether was added to the constant volume of the volumetric flask. After 2 min oscillation and extraction for 30 min, dissolved purple gallic element was extracted into the diethyl ether phase. Colorimetric estimation for purple gallic element was carried out using ultraviolet spectrophotometer at 430 nm. Purple gallic element quantity (milligram) from 1g soil after 1h indicated the activity of polyphenol oxidase.

Invertase was measured by sodium thiosulfate titration method (Yan, 1988) with minor modifications: 10g dried soils sample that had been through 1mm sieve were put into the volumetric flask. Then 1.5mL toluene was added into the volumetric flask. 15 min later, 10mL 20% sucrose solution and 10mL phosphate buffer (pH 5.5) was added into the volumetric flask, than the volumetric flask was put in incubator at 37° for 23 h. The volumetric flask was fed into constant volume (100mL) using distilled water of 38°. After fully mixed, the volumetric flask was kept in incubator for 1 h. After the incubation, the suspension was filtered with density of filter paper. Twenty mL filtrate mixed with 10mL protoporphyrin sodium solution and 20mL distilled water was boiled for 10 min, then suddenly cooled under running water to 25°. Then 3mL KI solution and 4mL dilute sulphuric acid (1:3) was added into the tube. The mixture was finally titrated using 0.1 mol·L^{-1} sodium thiosulfate. 0.5 mL starch indicator was added before reaching the terminal point, where the blue color just disappeared.

Microorganism number measurement: Bacteria were separated by beef extract peptone medium, and actinomyces was separated by Gauze's medium NO.1 supplemented with streptomycin, and fungus was separated by Martin medium supplemented with rose-bengal. The following steps were carried out on superclean bench. 10g of each soil sample was measured, and added into triangular flask containing 90mL sterile water, and soil solution of 10^{-1} concentration was obtained after 30 minute's oscillation. After standing for 1 min, 1mL soil solution of 10^{-1} concentration was mixed with 9mL sterile water using a pipetter and fully mixed, and the mixture was the soil solution of 10^{-2} concentration. By using the same method, soil solution was diluted to 10^{-5}, 10^{-4} and 10^{-3} for bacteria, actinomyces and fungus determining. Bacteria, actinomyces and fungus were inoculated onto the corresponding medium using dilution-plate method and 4 replicates were conducted. After inoculation, the culture dish was put upside down into the incubator at 28-30°. The number of the microorganism per g of dried soil was observed and counted. Water content measurement was as follows: soil sample was dried to constant weight in the drying oven at 105-110°. Three replicates were conducted for each treatment. Water content = (lost water weight / dried soil sample weight) ×100%. Number of microorganism/ g dried soil = (average number of microbial colony of several replicates under the same diluted time × diluted times) / (1-soil content).

Data analysis: RI was calculated by Williamson (Yu & Lu, 1988) method. RI=1-C/T T≥C or RI=T/C-1 T<C . C was control data, and T was treatment data. When RI was higher than zero, it means promotion, otherwise RI<0 means inhibition. The size of the absolute value of RI represented the degree of allelopathy.

The data was analyzed using software DPS, and significance of difference was tested using Duncan's new multiple range method.

Results

Effects of exudates and residues on Beta plant growth: Root exudates of low concentrations promoted the growth of seedlings. The lower of the concentration was, the more vigorous of the seedlings were. With the concentration increased, the growth of seedlings declined, and when the concentration reached 300mL (E300), the growth of the seedling was obviously depressed, where the plant height, stem diameter and fresh weight of shoot and root dropped dramatically by 25.82%, 14.58%, 28.08% and 19.11% respectively, compared with the control (Table 1).

The growth of seedlings was depressed when adding root pieces to soils (Table 2). With the increase of root adding proportion, the growth of seedlings was depressed. Plant height and stem diameter of three treatments were lower than the control, while only the difference between F5% treatment and the control reached significantly ($p<0.05$) level.

Table 1. Effects of root exudates on pot seedlings growth of Beta grape.

Treatment	Plant height (cm)	RI	Stem diameter (cm)	RI	Fresh weight of shoot (cm)	RI	Fresh weight of root (cm)	RI
CK	24.59±2.14c		0.3538±0.0137a		14.03±0.21c		5.60±0.30b	
E100	33.80±1.70a	0.272	0.3649±0.0215a	0.030	24.06±0.20a	0.417	8.21±0.20a	0.318
E200	30.19±2.43b	0.185	0.3558±0.0132a	0.006	21.73±0.20b	0.354	7.83±0.20a	0.285
E300	18.24±0.84d	-0.258	0.3022±0.0129b	-0.146	10.09±0.31d	-0.281	4.53±0.30c	-0.191

Note: CK means Control , and E100 means 100 mL root exudates +200 mL nutrient solutions, and E200 means 200 mL root exudates +100 mL nutrient solutions, and E300 means 300 mL root exudates. (The same below)

The significant different at 5% are marked by the small letters. If any letter marked in one column is the same as other one, it imples no different between them. They mean the same in the follow tables and figures

Table 2. Effect of root residues on pot seedlings growth of Beta grape.

Treatment	Plant height (cm)	RI	Stem diameter (cm)	RI	Fresh weight of shoot (cm)	RI	Fresh weight of root (cm)	RI
CK	13.62±4.02a		0.3220±0.0499a		9.72±0.31a		11.13±0.20a	
F1%	11.56±1.97ab	-0.151	0.2950±0.0339ab	-0.0839	8.76±0.30b	-0.0988	7.58±0.10b	-0.319
F3%	11.31±1.22ab	-0.170	0.2927±0.0424ab	-0.0910	6.99±0.20c	-0.281	7.08±0.20c	-0.364
F5%	10.66±1.76b	-0.217	0.2686±0.0285b	-0.166	6.38±0.20d	-0.344	6.77±0.20c	-0.392

As the root adding proportion increased, fresh weight of shoots were lower than the control by 9.9, 28.1 and 34.4% and fresh weight of roots were lower than the control by 31.9, 36.4 and 39.2%. The decrease in the fresh weight of roots were more than the fresh weight of shoots, which indicated that the root system firstly suffered from the injury of root residues, then the seedlings showed weak growth.

Effects of root exudates on rhizosphere soil available nutrients, enzyme activity and microorganism number

Effects of root exudates on soil nutrients and enzyme activity: There was no significant difference of soil alkali solution nitrogen content between root exudates treatments of different concentrations and the control (Table 3). Alkali solution nitrogen of E300 treatment was significantly higher than those of treatment E100 and E200. Low concentration treatments (E100 and E200) increased the content of available P and available K, while high concentration treatment (E300) decreased their contents. It implied that root exudates of low concentration might activate the soil available P and available K to some extent.

There existed difference of the activity of urease, invertase and polyphenol oxidase among the treatments. Specifically, there was no urease activity difference between treatments and the control. The invertase activity in treatments was lower than that of control, and the activity decreased as the concentration increased. Polyphenol oxidase activity also decreased as the concentration increased, and E100 and E200 treatments were higher than the control, and E300 treatment was lower than the control.

Effects of root exudates on soil microorganism number: Bacteria number decreased after root exudates treatments. Compared with the control, E100, E200 and E300 treatments decreased by 38.08, 58.24 and 65.82%. Actinomyces number showed a decrease-increase-decrease pattern, and the highest number was in E200 treatment, and the number of actinomyces in all treatments were lower than the control. In comparison with the control, fungus numbers of E100, E200, E300 treatments increased by 49.81, 106.49 and 200.27% (Fig. 1).

It would be seen from the above analysis that root exudates had remarkable impact on the number distribution of soil microflora. From the changes of soil microflora, bacteria/fungus, actinomyces/fungus showed a decreasing tendency with increase of treatment concentration. It implied that root exudates could invert the rhizosphere soil from “bacterial type” to “fungal type”.

Effects of root residues on rhizosphere soil available nutrients, enzyme activity and microorganism number

Effects of root residues on soil nutrients and enzyme activity: The content of alkali solution nitrogen and available P increased after adding root pieces into the soil, which had a positive correlation with root residues concentration. With the increase of root pieces in the soil, available K content also increased, while the content of all treatments were lower than that of the control (Table 4). These results indicated that root residues could increase the content of alkali solution nitrogen and available P, but brought down the content of available K.

There existed difference of the activity of urease, invertase and polyphenol oxidase among the treatment of root residues (Table 4). Specifically, urease activity was higher than the control, and the activity enhanced as the increasing concentration of treatment; while the activity of invertase and polyphenol oxidase decreased, and the activity declined along with the increasing concentration of treatment.

Table 3. Effect of grape root exudates on nutrient content and enzyme activity of rhizosphere soil.

Treatment	Available N ($mg \cdot kg^{-1}$)	vailable P ($mg \cdot kg^{-1}$)	vailableK ($mg \cdot kg^{-1}$)	Urease ($mg\ NH_3 \cdot g^{-1}$)	Invertase ($0.1Na_2S_2O_3\ mL \cdot g^{-1} \cdot 24h^{-1}$)	Polyphenol oxidase ($mg \cdot g^{-1} \cdot h^{-1}$)	pH
CK	108.50±0.00ab	15.34±0.00b	816.67±1.09c	0.0681±0.0004a	4.17±0.021a	0.0853±0.00b	6.41±0.01c
E100	106.75±0.00ab	16.32±0.15a	994.63±1.13a	0.0641±0.003a	4.14±0.15a	0.1115±0.014a	6.52±0.006b
E200	105.00±5.35b	16.25±0.18a	970.08±0.045b	0.0653±0.001a	4.03±0.058ab	0.1072±0.0045a	6.58±0.01a
E300	115.50±4.95a	14.82±0.18c	772.87±1.02d	0.0643±0.002a	3.90±0.087b	0.0678±0.0018b	6.37±0.006d

Table 4. Effect of grape root residues on nutrient content and enzyme activity of rhizosphere soil.

Treatment	Available N ($mg \cdot kg^{-1}$)	Available P ($mg \cdot kg^{-1}$)	AvailableK ($mg \cdot kg^{-1}$)	Urease ($mg\ NH_3 \cdot g^{-1}$)	Invertase ($0.1Na_2S_2O_3\ mL \cdot g^{-1} \cdot 24h^{-1}$)	Polyphenol oxidase ($mg \cdot g^{-1} \cdot h^{-1}$)	pH
CK	105.00±0.00d	16.25±0.34b	673.77±1.11a	0.063±0.004d	4.69±0.28a	0.1022±0.0097a	6.62±0.0058c
F1%	114.42±1.15c	19.29±1.62ab	533.56±1.12d	0.075±0.001c	3.13±0.17b	0.0985±0.013a	6.58±0.0252d
F3%	129.50±1.52b	21.33±3.08a	576.07±0.97c	0.095±0.002b	1.66±0.18c	0.0834±0.0044b	6.69±0.01b
F5%	144.67±1.01a	23.72±2.86a	664.88±2.02b	0.108±0.001a	1.05±0.07d	0.0828±0.0063b	6.73±0.00a

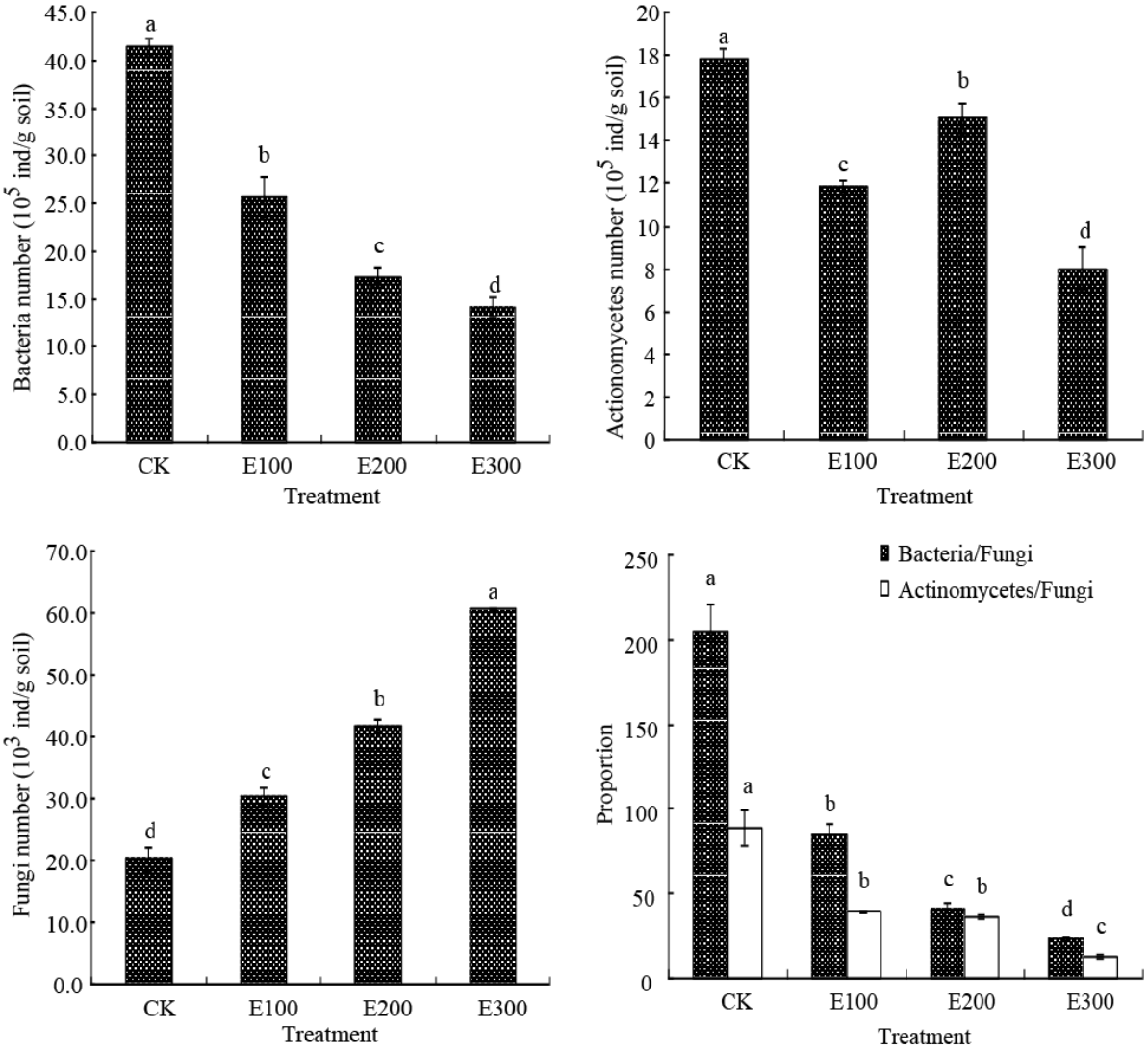

Fig. 1 Effect of grape root exudates on microbial number and proportion of rhizosphere soil

Effects of root residues on soil microorganism number: The number of bacteria, actinomyces and fungus increased with the increasing concentration of treatment (Fig. 2). Compared to the control, the number of bacteria increased by 132.40, 697.47 and 697.65% and the number of actinomyces increased by 85.37, 160.15 and 185.96% and the number of fungus increased by 41.06, 46.40 and 211.50%. Among them, bacteria number increased the most after root residues treatment.

As the increasing of root residues concentration, bacteria / fungus and actinomyces / fungus showed increase-decrease pattern. The highest value appeared in the treatment of F3% (Fig. 2).

As the major pathogen of plants, fungi's diversity decreased with the increasing of root adding proportion (Table 5). The beneficial *trichoderma* disappeared, while it appeared with *pythium*, *thielaviopsis* and *Stilbellales.*

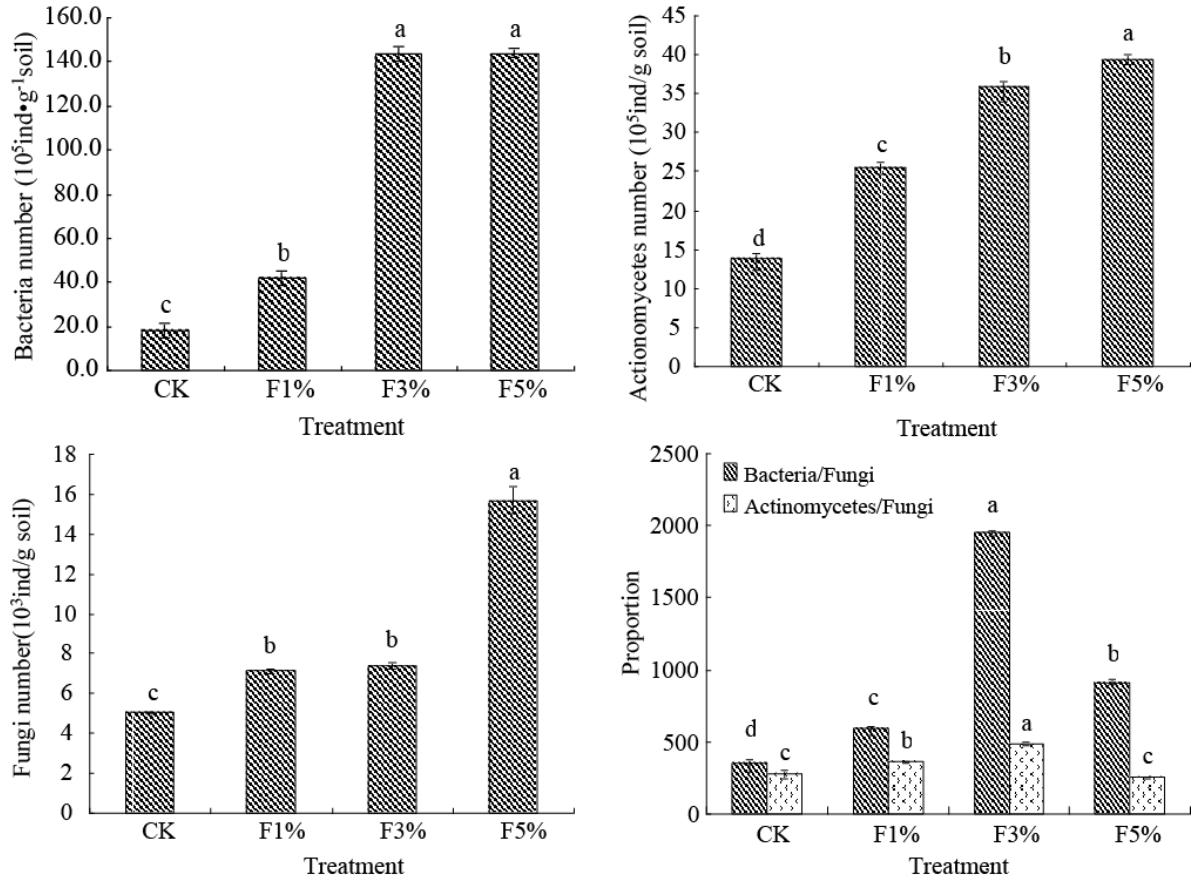

Fig. 2. Effect of grape root residues on microbial number and proportion of rhizosphere soil.

Table 5. Effect of grape root residues on fungal population.

Fungal population	CK	F1%	F3%	F5%
Trichoderma	+			
Monilia	+	+		
Alternaria	+	+		
Penicillium	+	+	+	
Cladosporium	+	+	+	+
Mucor	+	+	+	+
Paecilomyces	+	+	+	+
Pythium		+	+	+
Fusarium	+			
Aspergillus	+	+		
Gliocladium	+	+	+	
Rhizopus	+	+	+	+
Thielaviopsis			+	+
Stilbellales			+	+
Total	11	10	9	7

Note: '+ 'means the microorganism was isolated from rhizosphere soil

Discussion

Previous studies proposed that the deficiency or imbalance of rhizosphere soil nutrients was one of the major causes for grape replant disease (Li, 2010). Then do root exudates and residues have any effect on soil nutrients after a long-term planting? In this study, we have found that the content of soil available P and available K decreased and the alkali solution nitrogen content increased under the highest concentration treatment of root exudates (E300), and the plant growth was depressed. The content of available N, P and K increased as the addition ratio of root residues increased, and the plant growth was also depressed. The results implied that the rhizosphere soil nutrients changed under the treatment of root exudates and residues, however, this change was not the major reason that led to the growth depression of the plants. Further studies are needed to investigate the changes of micro elements as well as their relationship with the plant development as reported by Yoon-Ha Kim, *et al.,* (2012) and Kang *et al.,* (2012).

Urea hydrolyzes into ammonia under the catalysis of soil urease, which plays an important role in N utilization ratio and nitrogen recycle in soils. Sugar hydrolyzed into glucose and fructose under the catalysis of invertase, which plays an important role in increasing the nutrients which were easy to be dissolved. It was considered that the activity of invertase were greater with the increasing of soil fertility (Guan, 1986; Hamayun *et al.,* 2011). In this work, we've found that alkali solution nitrogen had some correlation with urease under the treatment of root exudates and residues. Invertase activity reduced as the root exudates and residues increased, and it had not any obvious correlation with plant growth or soil nutrition level.

Polyphenol oxidase converted the aromatic compound into quinone, which reacted with protein, amino acid, sugar and mineral in the soil, and generated organic matter and pigment with different molecular, thus finished the cycling of aromatic compound (Jia *et al.,* 1995; Trasar-cepeda *et al.*, 2000; Toscano *et al.,* 2003). So far, there existed 2 opposite standpoint about the correlation between soil polyphenol oxidase activity and plant growth. Zhang *et al.,* (2000) considered that polyphenol oxidase had some correlation with phenol acid accumulation, and that polyphenol oxidase activity was higher in replanted soybean soil than that in virgin soil, that was because the polyphenol oxidase activity was enhanced under the induction of phenol acid, and the polyphenol oxidase enhanced the activity of growth hormone oxidase in the plant which could decompose the growth hormone and affect the plant growth. Jia *et al*., (1995) believed that the stronger the polyphenol oxidase activity was, the less phenol content was in the soil, which was beneficial for plant dry matter accumulation, and vise versa. In this work, we've found that polyphenol oxidase decreased and the plant growth was also depressed as the root exudates and decompositions increased in the soil. This was consistent with Jia's point. This implies that the decrease of polyphenol oxidase activity resulted in the increase of phenol content in the soil, and the high content of phenol affected the seedlings growth.

It had found that soil biotic factor was one of the important reasons of grape replanting obstacle in previous soil sterilization experiment (Guo *et al.,* 2010) and that bacteria and fungus population structure changed after grape replanting (Guo *et al.,* 2011). But what lead to this change is still hanging in doubt. So far, root exudates are widely considered to be the core factor in rhizosphere micro-ecology. Its change led to the conversion of microorganism population (Felix & Donald, 2002; Harsh *et al*., 2006; Neeru *et al*., 2009; Chaudhry *et al*., 2012) which weakens or eliminates the antagonism between beneficial organism and pathogen (Qureshi *et al*., 2012). To investigate the relationship of grape root exudates and microorganism in rhizosphere soil, we carried out potted trail. The results showed that the ratio of bacteria / fungus and actinomyces / fungus decreased as the concentration of root exudates increased which meant that high concentration of root exudates could convert the rhizosophere soil from 'bacteria type' to 'fungi type'. It was consistent with some reports (Ma *et al.,* 2004; Zhao *et al.,* 2008) which considered that soils changed from high fertility 'bacteria type' to low fertility 'fungi type' soil after replanting. This indicated that grape root exudates not only inhibited seedlings growth directly, but also affected plant growth through regulating the quantity and composition of the microbiological flora in soils. In addition, root residues was an important reason of grape replanting obstacle, which would inevitably affect soil microorganism during the decomposing of root. In this work, the ratio of bacteria / fungus and actinomyces / fungus both increased under root residues treatments of different concentration. The diversity of fungus in rhizosphere soil decreased as the addition ratio of root residues increased. The beneficial *trichoderma* disappeared, while it appeared with *pythium*, *thielaviopsis* and *Stilbellales*. According to the report of Lou (2005), most of *pythiums* was plant pathogens which could lead to root rot, fruit decay and damping off. Westphal *et al*., (2002) also isolated *Pythium* spp. from replanted grape root. It was finally known from the above analysis that the change of microorganism after replanting was extremely complex. Both root exudates and residues could lead to the change of microorganism number and microflora, but the consequences were different. So, it was very important to deeply study the changing tendency of microorganism population in the rhizosphere soil after replanting and the factors that led to this change.

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