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# Reductive soil disinfestations combined or not with *Trichoderma* for the treatment of a degraded and *Rhizoctonia solani* infested greenhouse soil

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ABSTRACT

Intensive cultivation in greenhouses of China has degraded the soil through acidification, salinization, and has caused an increase in soil-borne pathogens accumulation that negatively impacts vegetable production. In this study, six treatments, i.e. CK, degraded soil collected from greenhouse was irrigated to maximum field capacity and covered with plastic film; Al-RSD-F, flooding soil incorporated with alfalfa; Al-RSD, soil was incorporated with alfalfa, irrigated to maximum field capacity and covered with plastic film; Al-RSD + T37, inoculating *Trichoderma harzianium* T37 into soil at the end of the Al-RSD; Et-RSD, soil was incorporated with ethanol, irrigated to maximum field capacity and covered with plastic film; AW, soil was incorporated with ammonia and covered with plastic film, were conducted to investigate their effects on the physicochemical properties of degraded soil and disinfestations of *Rhizoctonia solani*, to control the damping-off disease in the two consecutive plantings of cucumber seedlings after the treatments. Treatments with Al-RSD-F, Al-RSD, Al-RSD + T37 and Et-RSD increased the pH and reduced the electrical conductivity of the soils, reduced population of *R. solani* and damping off of cucumber seedlings. These treatments with exception of Et-RSD, increased the populations of bacteria and fungi and showed higher diversity of bacteria after the second season of cucumber seedlings. Treatment AW was toxic and did not allow the survival of the cucumber seedlings. The treatments Al-RSD + T37 and Et-RSD showed the higher control of damping off, probably because the antagonist was favored by the previous treatment and could efficiently colonize the cucumber seedlings roots, and the Et-RSD was favored by the highest accumulation of toxic acetic acid in the soil. Alfalfa-amended soil disinfestations with or without *Trichoderma*, as well as an ethanol soil disinfestation could be used to recover degraded greenhouse soils.



## 1. Introduction

Driven by economic benefits, the area for cultivating greenhouse vegetables has risen up to 24.8 million ha, occupying 14.5% of the farmland in China (Food and Agriculture Organization, 2013). Compared with cereal production, vegetable cultivation often adopts intensive management practices of over-fertilization, intensive irrigation and continuous mono-cropping (Power and Schepers, 1989). This degraded soil through acidification, salinization, and especially buildup of plant soil-borne pathogens population (Cao et al., 2004; Blok et al., 2000). Consequently, vegetable growth is seriously restrained, economic incomes of farmers are dramatically reduced, thus negatively affecting the sustainable development of greenhouse vegetable production. For example, cucumber damping-off, a seedling disease mainly caused by *Rhizoctonia solani* Kühn, universally occurs in the greenhouse cultivations of China and leads to serious loss in cucumber production (Li, 1995).

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Due to the increasing concerns about food safety and environmental pollution, the use of some traditional chemical fungicides has been gradually restricted (Adesina et al., 2007). For example, methyl bromide as an effective chemical fumigant had been forbidden in many countries, such as USA, Japan and China, according to the Montreal Protocol (Besri et al., 2010). Thus, in many cases, farmers have been forced to seek effective alternatives to manage soil-borne pathogens (Gamliel et al., 2000). Biological control of soil-borne diseases using microbial antagonists has become popular during the past decades (Huang et al., 2012). However, in general, the inoculated microbial strains do not survive well in the soil, which is essential to ensure their control efficiency (% disease control, CE) (Huang et al., 2012). In 2000, a new method, reductive soil disinfestation (RSD), developed separately in Japan (Shinmura, 2000) and The Netherlands (Blok et al., 2000) as an alternative to chemical soil disinfestation, became popular for its effectiveness in reducing soil inoculum levels of soil-borne pathogens and nematodes (Goud et al., 2004).

RSD based on supplying labile carbon to stimulate microbially driven anaerobic soil conditions in moist soils covered with polyethylene mulch (Butler et al., 2012) has been proven effective against a wide range of soil-borne pathogens, such as *Fusarium oxysporum* f. spp. *lycopersici*, *spinaciae* and *radicis-lycopersici*; *Fusarium redolens*; *Phomopsis sclerotioides*; *Pyrenochaeta lycopersici*; *Ralstonia solanacearum*; and *Verticillium dahliae*, as well as the nematodes *Meloidogyne incognita* and *Pratylenchus* sp. (Shinmura 2000, 2004). The possible mechanism of RSD involves the production of antagonistic compounds, such as organic acids (Huang et al., 2015), the released $Fe^{2+}$ and $Mn^{2+}$ (Momma et al., 2011), ammonia (Tenuta and Lazarovits, 2002) and hydrogen sulfide, by the functional anaerobic microorganisms. Apart from reducing the plant pathogens, RSD provides an environmental-friendly approach to deal with agricultural residue accumulation problems and decreases the costs associated with the use of chemical fertilizers and pesticides (PiedraBuena et al., 2006).

Although RSD is increasingly utilized in the USA,The Netherlands and Japan to control soil-borne diseases (Momma et al., 2013), the positive effect of RSD on the physicochemical properties of soil degraded by intensive cultivation has been scarcely studied (Zhu et al., 2012). Furthermore, few studies have investigated the changes of soil-borne pathogens and microbial communities during the consequent cultivations after RSD (Mowlick et al., 2013). Thus, various RSD treatments incorporated with solid plant residuals and liquid ethanol (widely used in Japan, Momma et al., 2013) were performed in this study to investigate their effects on *R. solani* population and on cucumber (*Cucumis sativus* L.) *R. solani* damping-off disease (considered as a seedling disease), and their influences on soil microbial communities. Further, the antagonist *Trichoderma harzianum* SQR-T37 (T37) was also combined with RSD to possibly increase disease control.

## 2. Materials and methods

### 2.1. Soil and strain

The soil (silty clay, loess parent material) used in this study was collected from a greenhouse located in Nanjing, Jiangsu Province, China. Excessive fertilization and continuous mono-cropping led to serious soil acidification, secondary salinization and the occurrence of damping-off. The collected soil had the following properties: pH 5.3, organic matter 21.3 g $kg^{-1}$ soil, total nitrogen 2.48 g $kg^{-1}$ soil, $NO_3^-$ 602 mg N $kg^{-1}$ soil, and *R. solani* $2.3 \times 10^8$ internal transcribed spacer (ITS) copies $g^{-1}$ soil. *T. harzianum* SQR-T37 (T37) was provided by Nanjing Agricultural University, which commonly shows antagonistic effect against *F. oxysporum* and *R. solani* (Huang et al., 2011; Chen et al., 2010).

### 2.2. Root colonization of T37

Cucumber seeds of the cultivar JinChun 5 were surface-sterilized in 2% NaClO for 3 min and 75% ethanol for 2 min, and then rinsed thrice in sterile water. The seeds were then germinated on 9-cm plates covered with sterile wet filter paper at 25 °C for 48 h. T37 mycelium was prepared according to the methods described by Huang et al. (2011). Autoclaved vermiculite was inoculated with the T37 mycelium to obtain final concentrations of 5 g fresh weight per kilogram of vermiculite. After inoculation, the germinated seeds were transferred to the vermiculite, irrigated with half-strength Hoagland medium and incubated in a growth chamber at 25 °C under a 16-h light regimen. After 15 days, the cucumber roots were washed with sterile distilled water. The root surfaces were observed under the Cryo-scanning electron microscope (Philips XL-30 ESEM, Eindhoven, The Netherlands). The preparation of samples was according to Craig and Beaton (1996).

### 2.3. Experimental design

The experiment was performed with the following six treatments:

(1) The soil collected from greenhouse irrigated to maximum field capacity (MFC, water/soil ratio 57.4%, w/w) and covered with plastic film (CK);
(2) flooding soil incorporated with 1% (w/w) alfalfa (*Medicago sativa*) meal (Al-RSD-F);
(3) irrigating soil to MFC, incorporating with 1% (w/w) alfalfa meal and covering with plastic film (Al-RSD);
(4) inoculating T37 (1.2 g fresh weight mycelium per kilogram soil) into soil at the end of the Al-RSD (Al-RSD + T37);
(5) irrigating soil to MFC, incorporating with 1% (w/w) ethanol and covering with plastic film (Et-RSD) and
(6) incorporating soil with 1% (w/w) ammonia water (25%) and covering with plastic film (AW).

The alfalfa meal (total organic carbon 399.1 g $kg^{-1}$, total nitrogen 13.5 g $kg^{-1}$) was finely chopped (particles size <2 mm) before use. The T37 mycelium was prepared as described above. These treatments were carried out in boxes of dimensions 25 × 25 × 5 cm each packed with 2.5 kg of soil. Each treatment contained three replicates. After 15 days of anaerobic treatment at 30 °C, the plastic films were removed and the soils in all treatments were air-dried for 4 days. Nine pre-germinated cucumber seeds were planted in each box. The cucumber seedlings were removed from the soil after 25 days, being cultivated with average day and night temperatures of 28 and 20 °C, respectively. Afterwards, the soil in the box was thoroughly mixed and a second cultivation of cucumber seedlings was planted in the same soil. The soil moisture and air humidity were maintained at 14–22% and 40–60% during the two growth seedling seasons in a growth chamber, respectively. Soil samples (100 g) in all spots were respectively collected at the end of the anaerobic treatment and after the first season, and the second seasons. The damping-off disease severity indices (DSI, which equals the number of dead plants in a replication divided by nine of the cucumber seedlings) determined at the end of each season. The CE was calculated as follows: CE of the treatment = (DSI in control – DSI in the treatment)/DSI in control. Furthermore, *R. solani* strains were isolated from the diseased cucumber seedlings, which certified that the damping-off was caused by *R. solani*.

**Table 1**
Primers and probe used in the experiments.

| Primers and probe[a] | Functions | Sequence (5′–3′)[b] | Reference |
|---|---|---|---|
| Eub338(F) | Bacterial quantification | ACTCCTACGGGAGGCAGCAG | Lane (1991) |
| Eub518(R) | | ATTACCGCGGCTGCTGG | Muyzer et al. (1993) |
| ITS1f(F) | Fungal quantification | TCCGTAGGTGAACCTGCGG | Gardes and Bruns (1993) |
| 5.8s(R) | | CGCTGCGTTCTTCATCG | Vilgalys and Hester (1990) |
| ST-RS1(F) | *R. solani* quantification | AGTGTTATGCTTGGTTCCACT | Lievens et al. (2005) |
| ITS4(R) | | TCCTCCGCTTATTGATATGC | Gardes and Bruns (1993) |
| ITS1S(F) | *T. harzianum* T37 quantification | TACAACCTCCAAACCCAATGTGA | López-Mondéjar et al. (2010) |
| ITS1R(R) | | CCGTTGTTGAAAGTTTTGATTCATTT | López-Mondéjar et al. (2010) |
| ITS1(P)[c] | | AACTCTTTTTGTATACCCCCTCGCGGGT | López-Mondéjar et al. (2010) |
| GC-U968(F) | Universal primers for bacterial 16 S rDNA | GC.-AACGCGAAGAACCTTAC | Zoetendal et al. (1998) |
| L1401(R) | | GCGTGTGTACAAGACCC | Zoetendal et al. (1998) |
| NS1(F) | Universal primers for fungal ITS region | GTAGTCATATGCTTGTCTC | White et al. (1990) |
| GC-Fungi(R) | | GC.-ATTCCCCGTTACCCGTTG | May et al. (2001) |
| GC(bacteria) | GC-clamp for DGGE | CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG | Nübel et al. (1996) |
| GC(fungi) | | CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC | May et al. (2001) |

[a] F, forward primer; R, reverse primer; P, TaqMan probe.
[b] A GC-rich sequence (GC.-) attached to the 5′ end of sequence is indicated.
[c] The fluorescent dye at the 5′ end of the probe is FAM (6-carboxyfluorescein) and the quencher dye at the 3′ end is TAMRA (6-carboxy-tetramethylrhodamine).

### 2.4. The measurement of soil electrical conductivity, pH and the content of acetic acid

Soil pH was measured in a 1:2.5 (v/v) soil/water ratio using a S220 K pH meter (Mettler-Toledo International Inc., Shanghai, China). The soil electrical conductivity (Ec) was measured at a 1:5 (v/v) soil/water ratio using a conductivity meter (DDS-320, Dapu Instrument Co., Ltd., Shanghai, China). The acetic acids content in the soil samples collected at the end of the anaerobic treatment was quantified by high-performance liquid chromatography (HPLC) (Waters eAlliance 2695, Milford, MA, USA) with the method described by Huang et al. (2015). The column XDB-C18 (4.6 × 250 mm, Agilent, Santa Clara, CA, USA) was used for the reversed-phase chromatography. Acetic acid was purchased from Sigma (USA) to be used as a standard.

### 2.5. Real-time polymerase chain reaction quantifications of R. solani, T37, bacteria, and fungi

The PowerSoil® DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) was used to extract soil DNA according to the manufacturer's instructions. The collected soil samples were stored at −20 °C until the extractions could be performed. Polymerase chain reaction (PCR) amplifications were performed in eight-well tubes with a total volume of 20 μl in each reaction on a CFX-96 thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). For the SYBR Green real-time PCR quantifications of bacteria, fungi and *R. solani,* each PCR reaction contained 2 μl of the target DNA extract, 10 μl of the SYBR Green premix Ex Taq (2×, Takara, Japan), 1 μl of forward primer and reverse primer (Eub338 and Eub 518 targeted at the 16S ribosomal DNA (rDNA) gene of bacteria, ITS1f and 5.8s targeted at the ITS gene of fungi, and ST-RS1 and ITS4 targeted at the ITS gene of *R. solani*; 10 μM, Table 1), and 6 μl of sterile distilled water. For the TaqMan real-time PCR quantification of T37, each PCR reaction contained 2 μl of the target DNA extract, 10 μl of Premix Ex Taq™ (2 ×, Takara), 0.4 μl of ITS1S and ITS1R targeted at ITS gene of T37 (10 μM, Table 1), 0.8 μl TaqMan probe ITS1 (10 μM, Table 1) and 6.4 μl sterile distilled water. The thermal cycling conditions were 2 min at 95 °C followed by 40 amplification cycles of, 10 s at 95 °C, 20 s at 53 °C, and 30 s at 72 °C for quantifying bacteria and fungi; and 10 s at 95 °C and 34 s at 60 °C for quantifying *R. solani* and T37. Fluorescence was detected at the third and second stages of each cycle. To evaluate amplification specificity, a melt-curve analysis was conducted at the end of each SYBR Green PCR run. The standard curves were developed according to López-Mondéjar et al. (2010). The slopes of the linear regression curve analyses were −3.00, −3.37, −3.40 and −3.15 for bacteria, fungi, *R. solani* and T37, respectively, which indicated very efficient amplification rates in the real-time PCR systems.

### 2.6. Denaturing gradient gel electrophoresis conditions

The microbial diversity of the soil samples was determined by denaturing gradient gel electrophoresis (DGGE). The soil DNA was amplified using specific primers (U968 + GC and L1401 for bacteria,

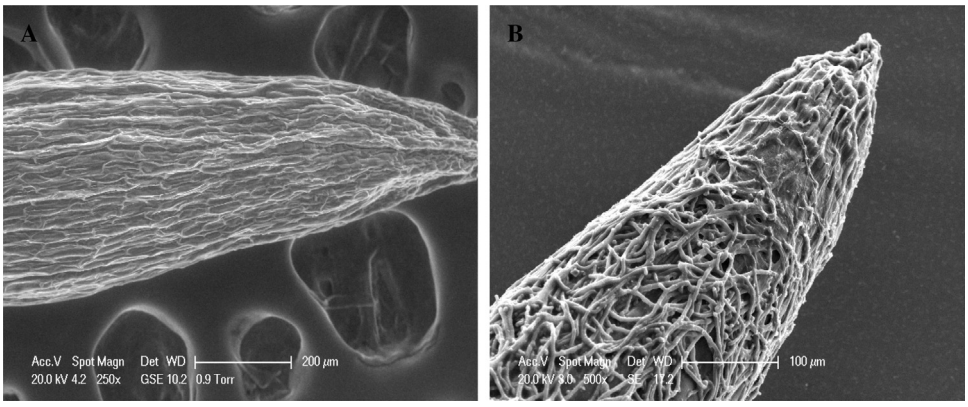


**Fig. 1.** Scanning electron micrographs of the colonization of *T. harzianum* T37 on cucumber root surface. (A), the untreated cucumber root. (B), the root of the cucumber planted in the vermiculite inoculated with *T. harzianum* T37 mycelium.

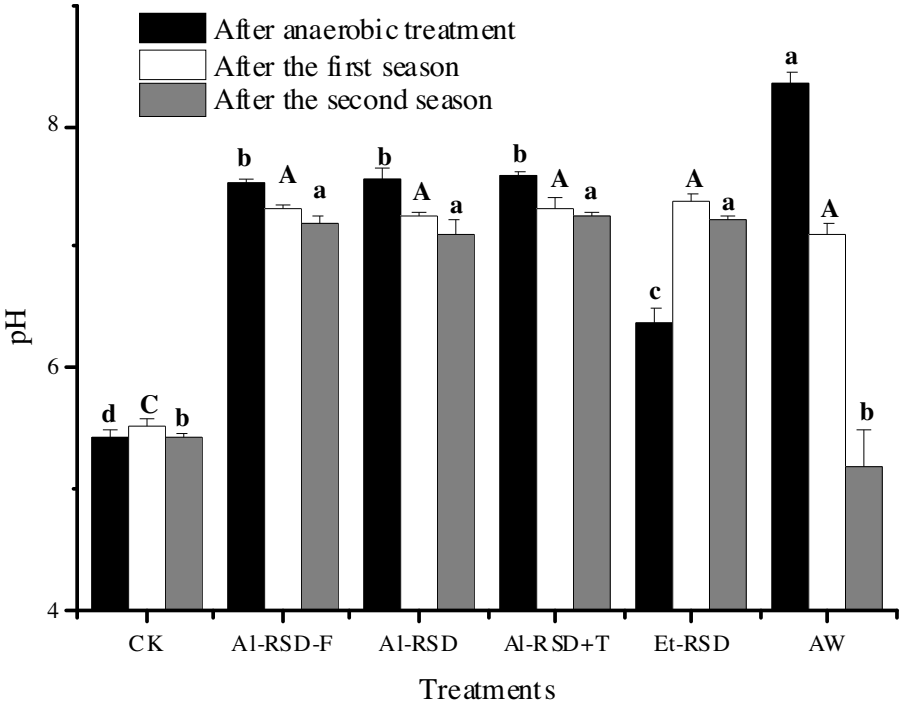


**Fig. 2.** Soil pH of different treatments at the end of anaerobic treatment, the first growth season, and the second growth season. CK, soil was irrigated to maximum field capacity (MFC, water/soil ratio 57.4%, w/w) and covered with plastic film; Al-RSD-F, flooding soil incorporated with 1% (w/w) alfalfa (*Medicago sativa*) meal; Al-RSD, the soil was incorporated with 1% (w/w) alfalfa meal, irrigated to MFC and covered with a plastic film; Al-RSD + T37, T37 (1.2 g fresh weight per kilogram soil) was inoculated into soil at the end of the Al-RSD treatment; Et-RSD, the soil was incorporated with 1% (w/w) ethanol, irrigated to MFC and covered with a plastic film and AW, the soil was incorporated with 1% (w/w) ammonia water (25%) and covered with a plastic film. Bars with different letters represent significant differences among the six treatments at the same time following Duncan's tests ($p < 0.05$). Error bars indicate standard deviations (SDs).

NS1 and Fungi + GC for fungi, Table 1), which amplified 490- and 370- bp fragments with a 40- bp GC- clamp, respectively. PCR was performed using 2.5 μl of 10 × Ex Taq buffer (20 mM $Mg^{2+}$, TaKaRa), 2 μl of 2.5 mM deoxyribonucleoside triphosphate (dNTP) mixture, 0.25 μl 5 units $\mu l^{-1}$ Ex Taq polymerase (TaKaRa), 1 μl of each primer (10 μM), 1 μl of soil DNA template, and double distilled (dd) $H_2O$ to a total of 25 μl. PCR was performed under the following cycling conditions: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, elongation at 72 °C for 45 s, and a final elongation step at 72 °C for 10 min. The products obtained from the bacterial and fungal PCR reactions were checked by agarose gel electrophoresis.

DGGE analysis was conducted using the Dcode Universal Mutation Detection System (Bio-Rad Laboratories Inc., CA, USA). The PCR products were loaded onto 6% (w/v) polyacrylamide gels (40% acrylamide/bis-solution, 37.5:1, Bio-Rad) with denaturing gradients ranging from 40% to 60% for the bacterial DNA and from 20% to 40% for the fungal DNA. The gels with bacterial and fungal DNA were run at 60 °C and 80 V for 16 h. Quantity One 4.6.3 (Bio-Rad) was used to analyze the digital images of the obtained gels.

### 2.7. Statistical analysis

The differences among all the treatments were assessed with one-way analysis of variance (ANOVA) at the end of each assay. Duncan's multiple range tests were applied when one-way ANOVA revealed significant differences ($P \leq 0.05$). SPSS version 13.0 was used for statistical analysis (SPSS Inc., Chicago, IL, USA). Species abundance, used as a proxy for the microbial communities, was defined as the number of DGGE-detected bands. The Shannon–Weaver diversity index ($H'$), which characterized the diversity of the dominant species in the soil microbial community (Shannon and Weaver 1963), was calculated as $H' = -\sum Pi \ln Pi$ based on the relative band intensities ($Pi$). The software Quantity One (version 4.6.3, Bio-Rad) and BIO-DAP (Resource Conservation Fundy National Park, Alma New Brunswick, Canada) were used to calculate the $Pi$ and H′ values, respectively. Principal Component Analysis (PCA) was performed with Canoco 4.5 (ter Braak and Smilauer, 2002).

**Table 2**
Soil electrical conductivities (Ec) and the concentrations of acetic acid (±standard deviation) in the soils after anaerobic treatment.

| Treatments | Ec ($\mu S\,cm^{-1}$) | Acetic acid ($mg\,g^{-1}$ soil) |
|---|---|---|
| CK | 683 ± 40b | 0c |
| Al-RSD-F | 429 ± 23d | 0.08 ± 0.03bc |
| Al-RSD | 475 ± 27d | 0.11 ± 0.01bc |
| Al-RSD + T | 476 ± 28d | 0.14 ± 0.03b |
| Et-RSD | 565 ± 14c | 1.48 ± 0.15a |
| AW | 1359 ± 93a | 0c |

CK, soil was irrigated to maximum field capacity (MFC, water/soil ratio 57.4%, w/w) and covered with plastic film; Al-RSD-F, flooding soil incorporated with 1% (w/w) alfalfa (*Medicago sativa*) meal; Al-RSD, the soil was incorporated with 1% (w/w) alfalfa meal, irrigated to MFC and covered with a plastic film; Al-RSD + T37, T37 (1.2 g fresh weight per kilogram soil) was inoculated into soil at the end of the Al-RSD treatment; Et-RSD, the soil was incorporated with 1% (w/w) ethanol, irrigated to MFC and covered with a plastic film and AW, the soil was incorporated with 1% (w/w) ammonia water (25%) and covered with a plastic film. Within columns, means followed by different letters are significantly different at $p < 0.05$ according to Duncan's tests.

## 3. Results

### 3.1. T37 colonization of cucumber root

Fifteen days after inoculation, the root surfaces of the cucumber inoculated with T37 were surrounded by T37, with a corresponding biofilm of T37 mycelium on the root surface of the plants (Fig. 1).

**Table 3**
Incidence of damping-off caused by *Rhizoctonia solani*.

| Treatments | Damping-off incidence (%) | | Shoot fresh weight of survival plants (g) | |
|---|---|---|---|---|
| | First season | Second season | First season | Second season |
| CK | 63.1 ± 23.1a | 100 ± 0a | 0.98 ± 0.22b | –[a] |
| Al-RSD-F | 25.9 ± 12.8b | 70.4 ± 6.4b | 0.98 ± 0.13b | 0.85 ± 0.22b |
| Al-RSD | 7.4 ± 6.4b | 33.3 ± 11.1c | 1.27 ± 0.17a | 1.06 ± 0.12a |
| Al-RSD + T | 7.4 ± 12.8b | 7.4 ± 12.8d | 1.41 ± 0.14a | 1.09 ± 0.11a |
| Et-RSD | 3.7 ± 6.4b | 7.4 ± 12.8d | 1.08 ± 0.14b | 0.91 ± 0.08b |
| AW | –[b] | | | |

Within columns, means followed by different letters are significantly different at $p < 0.05$ according to Duncan's tests.
[a] No data could be collected due to lack of seedling growth.
[b] No data could be collected due to phytotoxicity in the AW treatment.

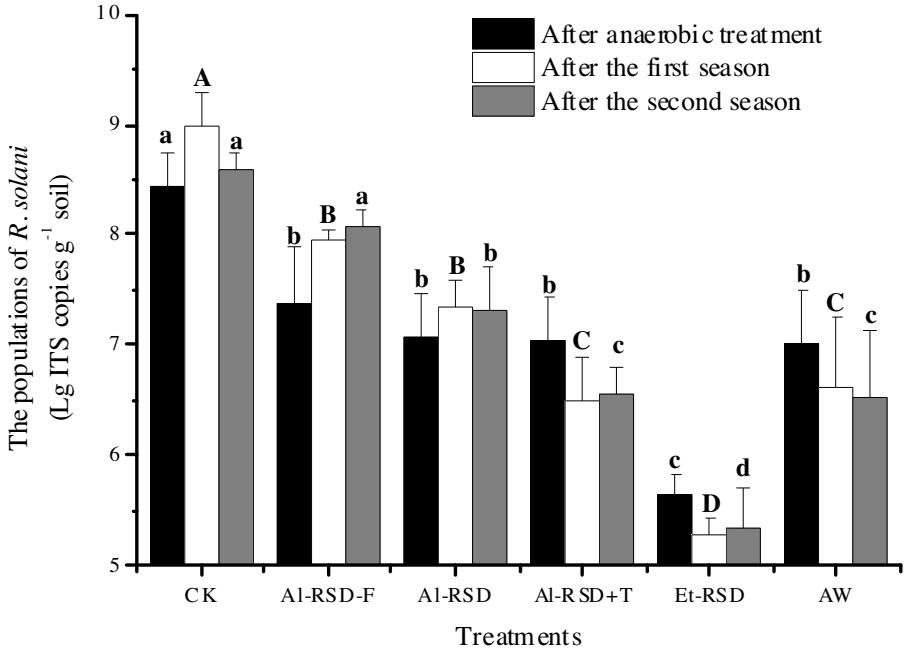


**Fig. 3.** Populations of *R. solani* in the different soils at the end of anaerobic treatment, the first growth season, and the second growth season. Bars with different letters represent significant differences among the six treatments at the same time following Duncan's tests ($p < 0.05$). Error bars indicate SDs.

### 3.2. Soil pH, electrical conductivity and the contents of acetic acid

#### 3.2.1. Soil pH

After a 15-day anaerobic treatment, the soil pH in the RSD-related treatments (Al-RSD-F, Al-RSD, Al-RSD + T37, and Et-RSD) and AW were significantly ($p < 0.05$) higher than that in CK (5.44), with the highest pH (8.35) found in the AW treatment. It is worth noting that the soil pH in the Et-RSD treatment (6.36) was significantly lower than those in the Al-RSD-related treatments (Al-RSD-F 7.54, Al-RSD 7.55, and Al-RSD + T37 7.59). At the end of the second growth season, the soil pH in the RSD-related soils was still significantly ($p < 0.05$) higher than that in CK. However, the pH in the AW treatment (5.20) further decreased, therefore not significantly different from that in CK (Fig. 2).

#### 3.2.2. Soil Ec

Compared with CK, the soil Ec in all RSD-related treatments significantly ($p < 0.05$) decreased at the end of anaerobic treatment. Moreover, the soil Ec in the Al-RSD-related treatments was significantly lower than that in the Et-RSD soil. The lowest soil Ec found in the Al-RSD-F treatment was 0.429 mS $cm^{-1}$, which respectively decreased to 62.8% and 75.9% of those in the CK and Et-RSD treatment. By contrast, the largest soil Ec was found in the AW treatment (Table 2).

#### 3.2.3. Acetic acids determination

Acetic acid was the primary volatile fatty acid (VFA) detected at the end of the anaerobic treatment, found in all of the RSD-related soils. The acetic acid concentration in the Et-RSD soil was much higher than those in the other RSD treatments (Table 2). Acetic acid was not detected in the CK and AW treatments.

### 3.3. The DSIs and growth tendencies of cucumber seedlings

The DSIs in the RSD-related treatments were significantly ($p < 0.05$) lower than that in the CK treatment in the first season. The lowest DSI (3.7%) was obtained in the Et-RSD soil, with a CE (control efficiency, % disease control) of 94.1%. In the second season, the DSIs in the CK, Al-RSD-F, or Al-RSD treatments significantly elevated compared with those in the first season. The CE of Al-RSD (66.7%) was significantly higher than that of Al-RSD-F (29.6%), and the best CEs (92.6%) were observed in both Al-RSD + T and Et-RSD treatments (Table 3).

In the two seasons of cultivations, the shoot fresh weights of cucumber seedlings in the Al-RSD and Al-RSD + T treatments were significantly ($p < 0.05$) larger than those in the other treatments. The largest shoot fresh weight was found in the Al-RSD + T treatment, with 1.41 and 1.09 g in the first and second season, respectively. The

AW treatment led to a high concentration of $NH_4^+ - N$ and thereby could not support the growth of cucumber seedling (Table 3).

### 3.4. *Populations of* R. solani, *T37, bacteria and fungi*

#### 3.4.1. R. solani *quantification*

After anaerobic treatment, the populations of *R. solani* in the RSD-related soils were significantly ($p < 0.05$) lower than that in the CK treatment. The numbers of *R. solani* in the Al-RSD ($1.59 \times 10^7$ gene copies $g^{-1}$ soil) and Et-RSD treatments ($4.60 \times 10^5$ gene copies $g^{-1}$ soil) decreased by 95.1% and 99.9% of that in the CK treatment, respectively. It is worth noting that, although not significantly different, the population of *R. solani* in the Al-RSD-F treatment was 2.10 times higher than that in the Al-RSD treatment. The AW treatment also significantly reduced the population of soil *R. solani* by 95.1% of that in the CK treatment. After the first season cultivation, the populations of soil *R. solani* in RSD-related treatments were still significantly ($p < 0.05$) lower than that in CK, with the lowest population of *R. solani* ($1.93 \times 10^5$ gene copies $g^{-1}$ soil) in the Et-RSD treatment. Furthermore, the number of *R. solani* in the Al-RSD + T treatment was significantly ($p < 0.05$) lower than those in the Al-RSD-F and Al-RSD treatments. Similar results were observed at the end of the second cultivation, except for an increased number of *R. solani* in the Al-RSD-F treatment and lack of significant difference with that in the CK treatment (Fig. 3).

#### 3.4.2. *T37 quantification*

After 25 days of the first-season cultivation, the population of inoculated T37 significantly ($p < 0.05$) decreased from initial $1.47 \times 10^8$ gene copies $g^{-1}$ soil (immediately after inoculation) to $3.83 \times 10^7$ gene copies $g^{-1}$ soil under the Al-RSD + T37 treatment. However, no further decrease was observed during the second cultivation, with a final constant population of $4.25 \times 10^7$ gene copies $g^{-1}$ soil at the end of the second season (Fig. 4).

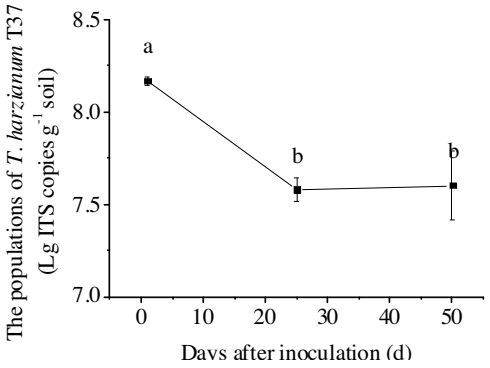


**Fig. 4.** Populations of *T. harzianum* T37 in the soils treated by the combination of RSD and *T. harzianum* T37 application at different times. Means with different letters represent significant differences following Duncan's tests ($p < 0.05$). Error bars indicate SDs.

#### 3.4.3. *Bacteria quantification*

After the anaerobic treatment, the populations of total bacteria in all RSD-related treatments were significantly ($p < 0.05$) increased compared with the CK and AW treatments. The largest number of bacteria found in the Al-RSD + T treatment was $1.38 \times 10^{11}$ gene copies $g^{-1}$ soil, about 4.46 or 2.31 times higher than that in the CK or Et-RSD treatment, respectively. Al-RSD-related soils exhibited obviously higher number of bacteria than the Et-RSD soils. Similar results were found at the end of the first season, except for the significantly increased number of bacteria in the AW soil than at the end of anaerobic treatment and lack of significant difference with that in the Et-RSD or Al-RSD treatment at this time. The population of bacteria in the Et-RSD treatment was significantly increased along with cultivation, being higher at the end of the second season, thereby not significantly different from other RSD treatments (Fig. 5).

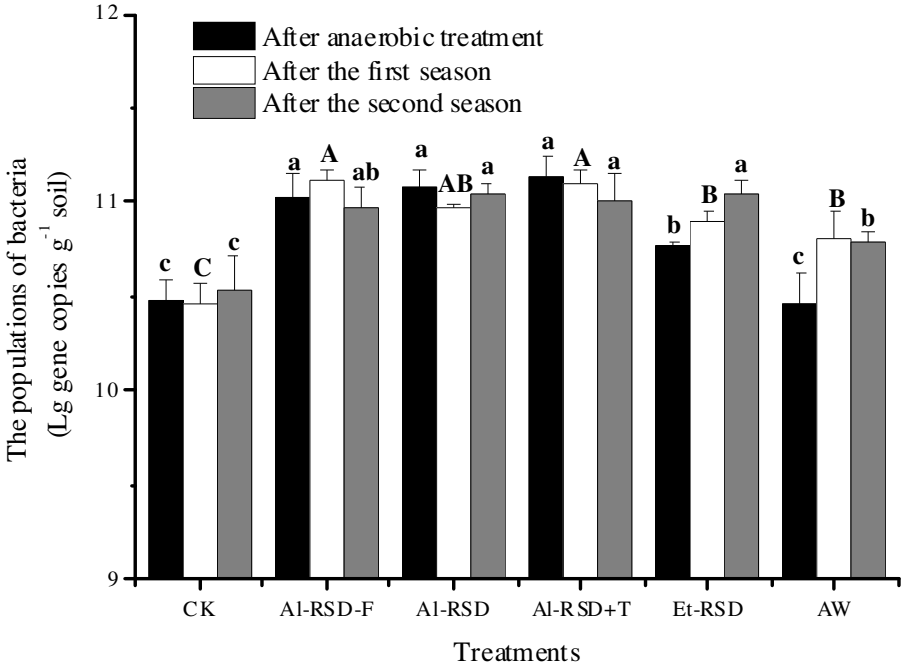


**Fig. 5.** Soil bacterial populations of different treatments at the end of anaerobic treatment, the first growth season, and the second growth season. Bars with different letters represent significant differences among the six treatments at the same time following Duncan's tests ($p < 0.05$). Error bars indicate SDs.

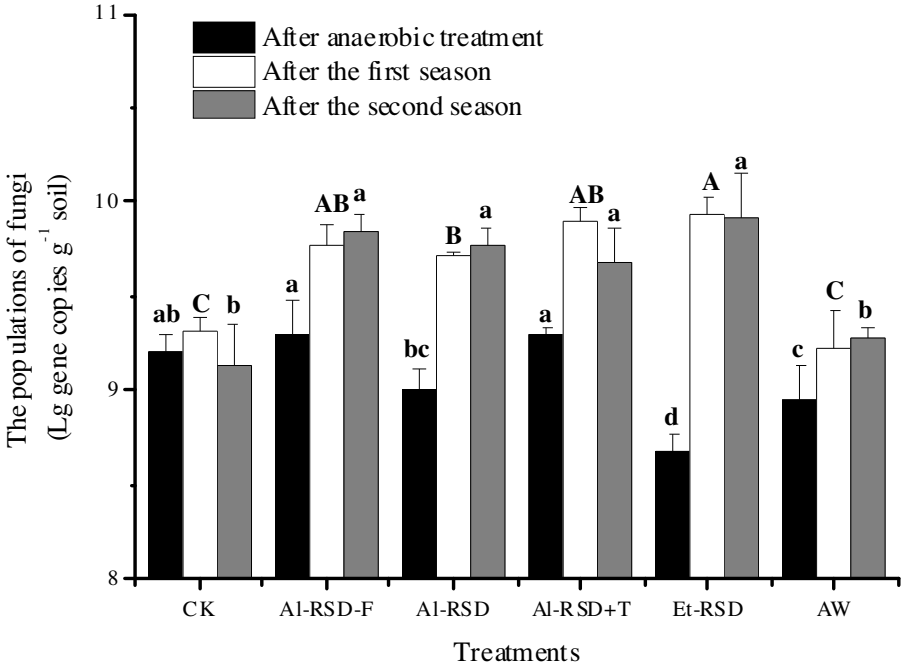


**Fig. 6.** Soil fungi populations of different treatments at the end of anaerobic treatment, the first growth season, and the second growth season. Bars with different letters represent significant differences among the six treatments at the same time following Duncan's tests ($p < 0.05$). Error bars indicate SDs.

**Table 4**
Proportions (±standard deviation) of *R. solani* in the different treatments at the three periods.

| Treatments | *R. solani*/Fungi (%) | | |
|---|---|---|---|
| | After anaerobic treatment | After the first season | After the second season |
| CK | 18.7 ± 9.7 | 56.7 ± 39.6 | 33.6 ± 19.9 |
| Al-RSD-F | 1.6 ± 1.3 | 1.6 ± 0.8 | 1.7 ± 0.5 |
| Al-RSD | 1.4 ± 1.2 | 0.5 ± 0.3 | 0.5 ± 0.6 |
| Al-RSD+T | 0.7 ± 0.5 | 0.1 ± 0 | 0.1 ± 0.1 |
| Et-RSD | 0.1 ± 0 | 0 ± 0 | 0 ± 0 |
| AW | 1.5 ± 1.4 | 0.6 ± 0.9 | 0.3 ± 0.4 |

Numbers followed by "±" are SDs.

#### 3.4.4. *Fungi quantification and* R. solani/*Fungi*

The populations of fungi in the Et-RSD and AW treatments were significantly ($p < 0.05$) lower than those in the other treatments after the anaerobic treatment. The lowest number of fungi was $4.83 \times 10^8$ gene copies $g^{-1}$ soil in the Et-RSD treatment, which was only 29.9% of that in the CK treatment. However, the populations of fungi in the RSD-related soils were obviously increased at the end of the first season, and being significantly higher than those in the CK and AW treatments. Similar results were noted at the end of the second season (Fig. 6).

All RSD-related treatments and AW treatment decreased the proportions of *R. solani* to fungi at the end of the anaerobic treatment. During the two cultivations, only the *R. solani*/fungi proportion in the CK treatment was increased. The proportions in the RSD-related treatments were decreased, except for the Al-RSD-F treatment with almost no change in the proportion (Table 4).

### 3.5. *The microbial communities*

The PCR–DGGE analyses of microbial communities (Fig. 7) revealed significant dissimilarities in both species composition (position of bands) and species diversity (number of occurring bands) among different treatment soils. Besides, microbial communities changed with time even in the same treatment.

After 15 days of anaerobic treatment, the bacterial abundances in the RSD-related soils became significantly ($p < 0.05$) higher than those in the CK and AW treatments, whereas the lowest abundance and diversities were found in the AW treatment. All Al-RSD-related treatments showed markedly higher bacterial abundances and Shannon diversity than the Et-RSD treatment. A similar phenomenon was observed at the end of the first season. Importantly, bacterial abundances and Shannon diversity in the Al-RSD and Al-RSD+T treatments were still noticeably higher than that in the Et-RSD soil. After the second cultivation, all RSD-related treatments significantly increased the bacterial abundances and Shannon diversity compared with the CK and AW treatments (Table 5). Furthermore, the bacterial community in Al-RSD soil was more closely related to the CK-treated soil than to the Et-RSD-treated soil (Fig. 8).

Compared with the CK treatment, the Et-RSD and AW treatments significantly decreased the fungal abundances and Shannon diversity at the end of the anaerobic treatment. After the first season, the fungal abundances and Shannon diversity in the RSD-related treatments were profoundly higher than those in the CK treatment. However, the fungal abundance and Shannon diversity were not found to be significantly different among the six treatments at the end of the second season (Table 6).

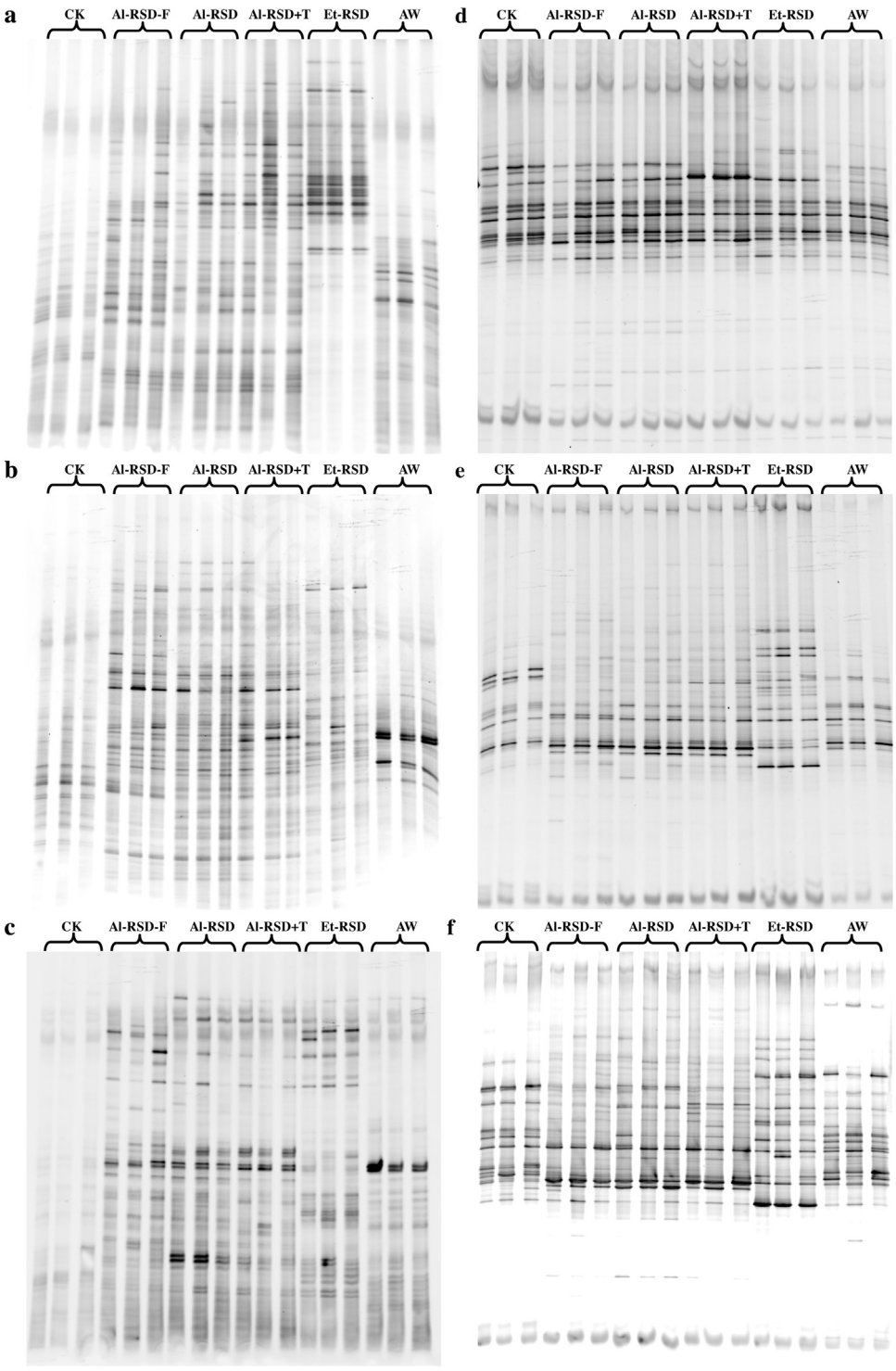


**Fig. 7.** Denaturing gradient gel electrophoresis profiles of bacterial (a, b, and c) and fungal (d, e, and f) communities in the soils of different treatments at the end of anaerobic treatment (a and d), the first season (b and e), and the second season (c and f).

## 4. Discussion

The soil used in this study was highly acidified (pH 5.3) due to the accumulations of nitrate (602 mg N $kg^{-1}$ soil), which is common in intensively cultivated greenhouses of China (Guo et al., 2010) and has negative influence on plant production (Shi et al., 2008). In this study, after anaerobic treatment, the soil pH significantly elevated in both RSD-related treatments and the AW treatment. It is generally known that the accumulated $NO_3^-$ is removed mainly through denitrification to gases under anaerobic condition (Burgin and Hamilton, 2005). The use of organic substrates in RSD could promote the removal of $NO_3^-$ in soil by stimulating microbial activities (Zhu et al., 2012), which might be responsible for the pH increases in the RSD-related treatments. Interestingly, the pH increase in the Et-RSD soil was less than those in the Al-RSD-related soils at the end of the anaerobic treatment. Momma et al. (2013) reported that organic acids, such as acetic acid and butyric acid, were accumulated during the RSD process. Because Et-RSD treatment produced

**Table 5**
Shannon diversities and abundances (±standard deviation) of soil bacteria in different treatments.

| Treatments | After anaerobic treatment | | After the first season | | After the second season | |
|---|---|---|---|---|---|---|
| | Shannon Diversity | Abundance | Shannon Diversity | Abundance | Shannon Diversity | Abundance |
| CK | 2.26 ± 0.04b | 10.67 ± 0.58c | 2.56 ± 0.02c | 17.00 ± 0.00c | 1.20 ± 0.11c | 4.33 ± 0.58c |
| Al-RSD-F | 2.74 ± 0.19a | 19.00 ± 2.65a | 3.01 ± 0.01a | 26.00 ± 7.81ab | 2.71 ± 0.10a | 21.33 ± 1.15a |
| Al-RSD | 2.65 ± 0.10a | 18.33 ± 0.58a | 3.13 ± 0.03a | 30.67 ± 1.53a | 2.79 ± 0.10a | 20.33 ± 2.08a |
| Al-RSD + T | 2.80 ± 0.09a | 20.67 ± 1.53a | 2.99 ± 0.14a | 28.67 ± 2.08a | 2.71 ± 0.20a | 20.00 ± 2.65a |
| Et-RSD | 2.45 ± 0.04b | 15.67 ± 1.15b | 2.77 ± 0.13b | 21.00 ± 2.00bc | 2.73 ± 0.06a | 19.67 ± 0.58a |
| AW | 1.96 ± 0.14c | 9.67 ± 0.58c | 1.62 ± 0.17d | 6.67 ± 0.58d | 2.18 ± 0.38b | 14.33 ± 0.58b |

Within columns, means followed by different letters are significantly different at $p < 0.05$ according to Duncan's tests.

**Table 6**
Shannon diversities and abundances (±standard deviation) of soil fungi in different treatments.

| Treatments | After anaerobic treatment | | After the first season | | After the second season | |
|---|---|---|---|---|---|---|
| | Shannon Diversity | Abundance | Shannon Diversity | Abundance | Shannon Diversity | Abundance |
| CK | 2.55 ± 0.03a | 20.67 ± 0.58a | 1.94 ± 0.09b | 10.00 ± 1.00d | 2.54 ± 0.05a | 15.33 ± 0.58a |
| Al-RSD-F | 2.48 ± 0.19ab | 22.00 ± 3.57a | 2.18 ± 0.04a | 13.67 ± 1.15bc | 2.48 ± 0.06a | 15.33 ± 1.15a |
| Al-RSD | 2.56 ± 0.06a | 18.67 ± 0.58ab | 2.19 ± 0.10a | 14.67 ± 2.89ab | 2.48 ± 0.08a | 15.67 ± 1.53a |
| Al-RSD + T | 2.36 ± 0.06b | 18.33 ± 0.58ab | 2.19 ± 0.11a | 15.33 ± 1.15ab | 2.46 ± 0.04a | 15.67 ± 0.58a |
| Et-RSD | 2.33 ± 0.09b | 16.00 ± 1.00b | 2.29 ± 0.09a | 16.67 ± 1.15a | 2.46 ± 0.04a | 14.00 ± 1.00a |
| AW | 2.34 ± 0.04b | 15.33 ± 0.58b | 2.01 ± 0.12b | 11.33 ± 0.58 cd | 2.49 ± 0.06a | 14.00 ± 1.00a |

Within columns, means followed by the different letters are significantly different at $p < 0.05$ according to Duncan's tests.

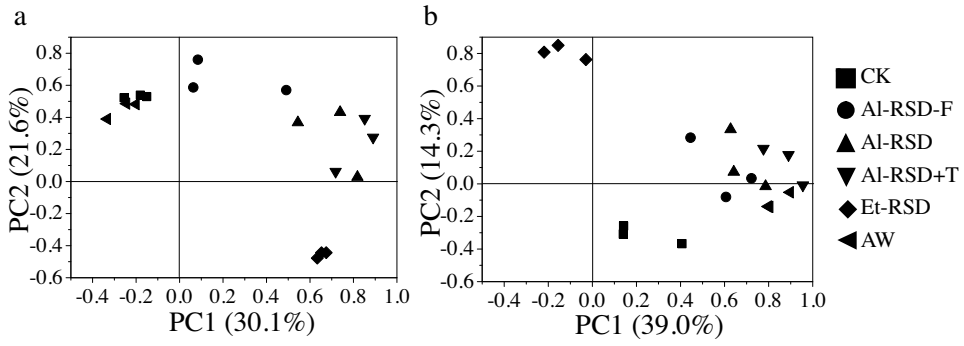


**Fig. 8.** Principal component analysis (PCA) of the bacterial communities in the soils at the end of the anaerobic cultivation (a) and the second season (b).

more acetic acids than the Al-RSD-related treatments, soil pH in Et-RSD was lower than in Al-RSD-related soils (Table 2) at the end of the anaerobic treatments. Then, the accumulated acetic acid in the Et-RSD soil was quickly decomposed and volatilized (Huang et al., 2015), which was verified by the significant increase in pH at the end of the first cultivation during Et-RSD treatment. By contrast, the soil pH in the AW treatment decreased below its initial level after the two cultivation seasons, possibly due to the accumulation of nitrate through the nitrification of the added ammonia.

In addition to acidification, salinization is also a problem caused by over-fertilization in intensive vegetable cultivation in China (Zhu et al., 2011). It is known that salinity (high Ec) reduces yield. RSD significantly reduced the soil Ec in this study, possibly via the microbially driven transformations of $NO_3^-$, $SO_4^{2-}$ and other ions to gases (e.g., $N_2$, $N_2O$ and $H_2S$) (Compeau and Bartha, 1985). In the present study, Al-RSD-related treatments reduced soil salinization better than Et-RSD did, possibly because broad-spectrum organic carbon sources in alfalfa stimulated higher microbial activities compared with the single C source in ethanol (Fontaine et al., 2003). Moreover, some white salt grains were found on the Et-RSD soil surface at the end of the anaerobic treatment, explaining the lower growth tendency of cucumber in the Et-RSD soil during the two cultivation seasons (Grattan and Grieve, 1999). Besides, the cucumber seeds could not grow well under the AW treatment due to its high concentration of $NH_4^+$.

Continuous mono-cropping is another feature of intensive vegetable cultivation in China, resulting in the enrichment of soil pathogens via the specific attractions of mono-crop root exudates (Ling et al., 2013). As a generally known alternative to chemical fumigations, RSDs are being increasingly used to suppress soil-borne pathogens through anaerobic decompositions of organic matters and the productions of antagonistic substrates, such as ammonia (Tenuta and Lazarovits, 2002), organic acids (Huang et al., 2015), $Fe^{2+}$, and $Mn^{2+}$ (Momma et al., 2011). In this study, ammonia fumigation displayed a similar disinfestation effect to Al-RSD, which is in agreement with previous report (Tenuta and Lazarovits, 2002). However, ammonia fumigation should be used sparingly as its high concentration of $NH_4^+$ can cause damage to plants. The disinfestation effect of Al-RSD-F was not as profound as Al-RSD. The possible reason was that the water layer above the soil was only 0.5–1 cm deep during the Al-RSD-F treatments, which could not entirely prevent the gaseous interchange from the atmosphere to the surface of the submerged soil, consequently reducing the accumulation of antagonistic compounds. This is very important when applying RSD in fields. Furthermore, Et-RSD showed a better disinfestation effect than Al-RSD as the former produced more acetic acid, which is consistent with our previous report (Huang et al.,

2015). Most interestingly, the application of T37 at the end of Al-RSD treatment further decreased the population of *R. solani* during the cultivations. Many researchers reported that the application of antagonists in the soil could suppress soil pathogens on the premise that they could compete with indigenous microorganisms, survive well and execute their specific functions (Jagnow et al., 1991; Van Veen et al., 1997). However, on direct application, in some experiments these antagonists could not survive well and compete with indigenous microorganisms (Huang et al., 2011; El-Hassan and Gowen, 2006). Here, the T37 population slightly decreased after the inoculation but remained constant during the second cultivation season. One of the possible explanations was that RSD disintegrated the soil microbial communities by transforming them from aerobic to anaerobic. When the soil was reexposed to aerobic conditions during the cultivations, an ecological vacuum appeared due to the rapid response of strictly anaerobic microbes to oxygen (Finlay et al., 1997). When introduced, T37 can potentially colonize this ecological niche, becoming more competitive and active, and ultimately further decrease *R. solani* population. Another explanation was that the added organic matters during RSD provided nutrient for the antagonists and helped them survive in the soil, which was in consistent with previous report (Huang et al., 2011) that the combined applications of antagonist and organic substrates could maintain the antagonist population and obtain a more efficient control on the soil-borne disease than individual application of antagonist. Furthermore, the effective colonization of T37 on cucumber root observed in this study might play an important role in suppressing the damping-off disease (Demoz and Korsten, 2006). Considering the re-increase of pathogen populations is a common concern when applying RSD, this problem can be overcome by introducing antagonists alongside RSD (through the fermentation of pure antagonist or the application of organic fertilizers).

A previous study (Chinn et al., 1962) reported that the populations of pathogens in soils were generally correlated to the DSI, which is consistent with the results obtained from this study. The CE of Al-RSD treatment in the second cultivation was less than that in the first season due to the increased *R. solani* population. By contrast, the Al-RSD + T and Et-RSD treatments promptly prevented cucumber damping-off disease in both cultivations as they suppressed the *R. solani* population better. Many researchers have reported that plant diseases can be successfully suppressed by effective colonization by biocontrol agents (Huang et al., 2011), which was in consistent with this study. Generally, a biocontrol efficiency of 50% is acceptable for application in the field (Minuto et al., 2006). Thus, RSD may serve as an effective alternative to reducing the occurrence of soil-borne disease, which in combination with the application of antagonists will achieve a stable control effect.

Soil microbial communities play important roles in controlling soil fertility and plant yields (Pankhurst and Lynch, 2006). Although the correlation between microbial diversity and function in soil is largely unknown (Torsvik and Øvreås, 2002), microbial diversity and activity are closely related to ecosystem function (Finlay et al., 1997), and higher microbial diversity has been assumed to induce a stable ecosystem and diversified functions, such as nutrient cycling and plant growth promotion (Torsvik and Øvreås, 2002). Generally, RSD treatments increased soil microbial, especially bacterial, populations and diversities compared with the CK and AW treatments in this study. More specifically, the Et-RSD treatment induced larger change in microbial community than Al-RSD-related treatments did. Alterations in the microbial community composition may lead to changes in microbial functions and overall soil quality (De Boer et al., 2003). Comparatively, Al-RSD showed a milder effect on the original microbial communities and functions (Mowlick et al., 2013). The Et-RSD and AW treatments decreased microbial diversities via their broad-spectrum disinfestation effect on soil microorganisms, and the loss of some microbial biomass and species possibly led to the loss of some microbial functions (Mowlick et al., 2013). However, the information obtained from DGGE profiles is limited, further works using molecular biology methods, such as high-throughput sequencing and metatranscriptomics, are still needed to investigate the detailed soil microbial communities and functions changes before and after these reductive soil disinfestations.

## 5. Conclusion

In a greenhouse degraded soil, alfalfa- and ethanol- amended reductive soil disinfestations reduced *Rhizoctonia solani* population, effectively controlled the damping off of cucumber seedlings, increased bacterial and fungal populations and improved the diversity of bacteria. The treatments Al-RSD + T37 and Et-RSD showed the higher control of damping off, probably because the antagonist was favored by the previous treatment and could efficiently colonize the cucumber seedlings roots, and the Et-RSD was favored by the highest accumulation of toxic acetic acid in the soil. Generally, alfalfa-amended soil disinfestations with or without *Trichoderma*, as well as an ethanol soil disinfestation could be used to recover degraded greenhouse soils.

## Acknowledgments

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