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Influence of pulsed electric field on growths of soil bacteria and pepper plant

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Abstract−DC 20 volt of electricity was charged to the electrodes placed around hot pepper plants to induce electrical redox reaction. Anode and cathode were periodically exchanged at intervals of 30 seconds to develop a pulsed electric field (PEF), by which the ORP of the soil around the pepper plant roots were fluctuated from 17 to −13 volts. Mean viable cell number of the intrinsic bacteria in five different positions was variable from 77,000 to 396,000 around electrodes and 339,000 to 680,000 around plants in the PEF, and 538,000 to 927,000 around plants in the conventional field. The mean viable cell number of the extrinsic bacteria (*R. solanacearum*) in five different positions was variable from 15,000 to 47,000 around electrode and 152,000 to 374,000 around plant in the PEF, and 294,000 to 607,000 around plants in the conventional field. Mean 3.93 and 5.67 of hot pepper plants were infected with bacterial wilt every two days by passive and active infection, respectively, in the conventional field. Mean 1.25 and 2.5 of hot pepper plants were infected with bacterial wilt every two days by passive and active infection, respectively, in the PEF. Mean sprouting number of seeds in the PEF and conventional field and was 45.0 and 48.2, respectively. Mean dry weight of hot pepper plants was 3.15 g and 2.51 g in the PEF and conventional field, respectively. The TGGE band pattern in the PEF was not very different in comparison with that in the conventional field (B and D) based on the band number, which corresponds to the bacterial diversity. This study suggests that the PEF would be functioning as an environmental factor to inhibit bacterial growth rather than to be a physical agent to destroy bacterial cells.

Key words: Pulsed Electric Field, *Ralstonia solanacearum*, Bacterial Wilt, Hot Pepper Plant, Oxidation-reduction Potential

INTRODUCTION

Ralstonia solanacearum has been known to infect over 450 plant species including numerous commercially valuable crops [1-3]. Extracellular polysaccharides produced by *R. solanacearum* are major virulence factors to cause the agriculturally important bacterial wilt [2,4]. The exopolysaccharide interferes with water transport in the plant by plugging the xylem vessels, leading to wilt [5]. Various studies have been performed to control the bacterial wilt; however, such investigations frequently gave promising results under *in vitro* or controlled conditions but have met limited success [6-9].

R. solanacearum, listed as a quarantine organism in the European Union (EU), is an object to be controlled and eradicate, for which new legislation was introduced [10]. Latent infections in seed potato tubers have led to the spread of the organism, both locally and internationally, which requires a rapid, specific, and sensitive detection assay to level lower than that occurring in naturally infected potatoes [11]. Practically, immunofluoroscence microscopy, the enzyme-linked immunosorbent assay, and molecular techniques involving the PCR have been described for detection of *R. solanacearum* [12-15]. However, presumptive cultures isolated on a semi-selective medium are required to identify the presence of the pathogen, and a host test on crop seedlings is required to confirm the pathogenicity [16,17]. The previous detection of pathogen to seeding or seedling can be a way to protect infection of crops with bacterial wilt; however, the infection from intrinsic pathogens in soil is difficult to protect. Some antibiotics or bacteriocin-producing bacteria were introduced to inhibit *R. solanacearum* within rhizosphere or induce host plant resistance. However, the poor competition ability and poor edaphic adaptation of the introduced bacteria are cited as the most probable causative explanation for limited success of bacterial antagonist [18-21].

Flow of direct-circuited electric charge through soil containing water can be generated between anode and cathode, which can be converted to PEF by periodic exchange of electrode poles [22,23]. The weak electric pulse was reported to inhibit the development of biofilm growing on carbon electrode and inactivate coliforms [24]. Giladi et al. [25] reported that the combined effect of the electric field and chloramphenicol increased the efficacy of antibiotics against bacterial film. In recent research, the PEF with very high intensity (2.0 to 5.0 V/μm or 16.7 kV/cm) effectively inactivated gram-negative, gram-positive and eukaryotic microorganisms in the low temperature (40 °C) condition [26] or in the condition containing low antibiotics concentration [27]. However, the PEF generated by high electric voltage is limited to be used in a specially designed bioreactor for inactivation or destruction of microorganisms [28,29].

In this study, we applied very low electric voltage to a hot pepper plant field to estimate the effect of the PEF on the growth of soil bacteria and *R. solanacearum*.

MATERIALS AND METHODS

1. Localization of Electrodes

DC 20 volts of electricity was charged to titanium electrodes (50 cm×0.5 cm) driven into field soil beside hot pepper plants and their

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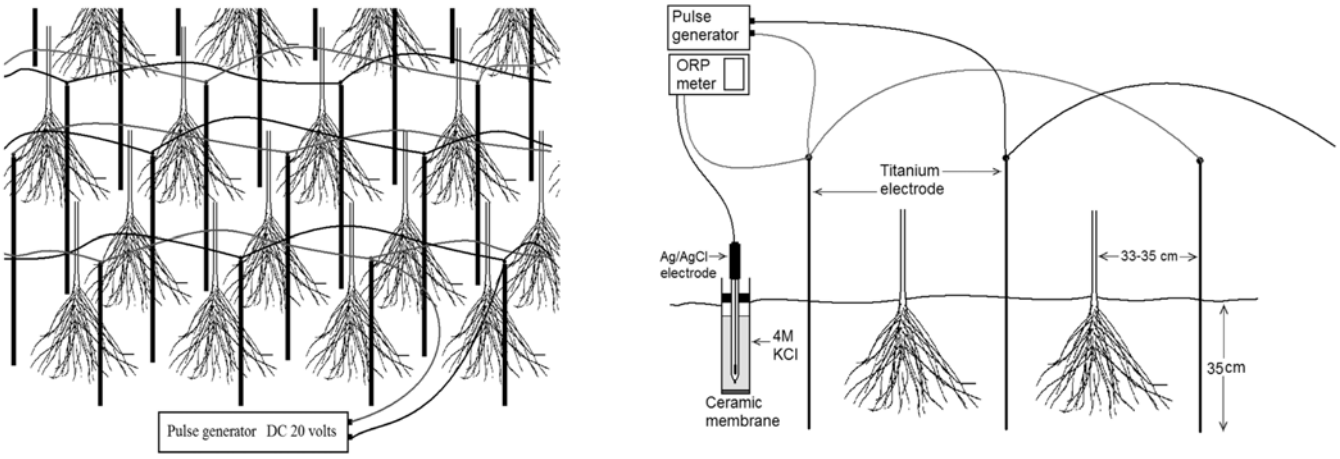

Fig. 1. Schematic diagrams for electrode position in hot pepper plant field (left). Electric pole of anode (black lines) and cathode (gray lines) was periodically exchanged at the intervals of 30 s. Four electrodes were disposed around one plant. The electric pulse generated by exchange of electric poles was measured with Ag/AgCl reference electrode (right).

buried depth was adjusted to 35 cm as shown in Fig. 1. The distance between electrode and plant was adjusted to 33-35 cm and the diagonal distance between electrodes was 66-70 cm. The unit electric intensity was calculated based on the electric voltage charged to electrodes and the distance between electrodes was as follows: 20 V÷(66-70)cm=0.286-0.303 V/cm. Anodic and cathodic power lines were alternately connected to the electrodes, and electrode poles were periodically exchanged at the interval of 30 seconds, by which PEF was developed. ORP variation of the PEF was measured by Ag/AgCl reference electrode.

2. Effect of PEF on Bacterial Growth

Field soil was sampled from five different sites in the PEF and conventional field. Two grams of soils sampled in 15-20 cm of depth were suspended in 9 mL of saline and vigorously mixed with vortex mixer for 3 min. The soil suspension was properly diluted by 10-fold dilution technique and then spread on plate count agar containing 0.005% of 2,3,5-triphenyl tetrazolium chloride (indicator) to separate *R. solanacearum* from other bacterial species. Viable cell number was expressed as the colony-forming unit (CFU: cell number/g dry soil).

3. Effect of PEF on Sprouting

Seeds of hot pepper plants were sown in the PEF and conventional field that were not contaminated with *R. solanacearum*. The sprouting number was daily counted in each field. After being cultivated for 50 days the hot pepper plants were rooted out, cleaned and completely dried at 100 °C for two days. Hot pepper plant growth was determined based on dry weight.

4. Effect of PEF on Wilt Protection

R. solanacearum was previously cultivated in the LB broth for 48 hrs. Hot pepper plants previously cultivated in uncontaminated flowerpots were transplanted to the PEF and conventional field and then 10 times diluted *R. solanacearum* culture was sprinkled on surface of field soil to induce passive infection. Meanwhile, the active infection was induced by pricking soil around roots with 10 cm of cutter knife. The sprinkle of bacterial culture was performed on the 3^{rd} and 6^{th} day after transplantation, and the sprinkle volume was adjusted to 100 mL/m^2. Initial number of hot pepper plants was adjusted to 150 in each test group. The infected hot pepper plants with bacterial wilt were counted after 10 days of the transplantation, which was specified as the zero incubation time when analyzed. The sap extracted from the infected plants with wilt disease was cultivated on plate count agar containing 0.005% of 2,3,5-triphenyl tetrazolium chloride for the final decision on diseased plants.

5. Temperature Gradient Gel Electrophoresis

A part of the soils sampled for bacterial cell count was used for the DNA extraction. DNA was extracted from the soils with a DNA extraction kit (PowerSoil™, Mobio, USA) in accordance with the procedure specified by manufacturer. Variable region of 16S-rDNA was amplified with forward primer (eubacteria, V3 region) 341f 5′-CCTACGGGAGGC AGCAG-3′ and reverse primer (universal, V3 region) 518r 5′-ATTACCGCGGCTGCT GG-3′. GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG GGGCCTACGGGAGGCAGCAG-3′) was attached to the 5′-end of the GC341f primer [30]. The TGGE system (Bio-Rad, Dcode™, Universal Mutation Detection System, USA) was operated as specified by the manufacturer. Aliquots (45 μl) of PCR products were electrophoresed in gels containing 8% acrylamide, 8 M urea, and 20% formamide with a 1.5×TAE buffer system [31] at a constant voltage of 100 V for 20 hrs, applying a thermal gradient of 39 to 52 °C.

6. Amplification of TGGE Band

DNA was extracted from TGGE band, and purified using a DNA gel purification kit (Accuprep, Bioneer, Korea). The purified DNA was amplified with the same primers and procedures used for TGGE sample preparation, in which the GC clamp was not attached to the forward primer. The amplified DNA was directly sequenced upon request to a professional company (Macrogen Inc., Republic of Korea). The partial 16S-rDNA sequences were analyzed using the GenBank database, and identification was performed based on 16S rDNA sequence homology.

7. Statistical Analysis

Multiple tests were carried out in all experiments and mean values, standard deviation and significance probability (*P*-value) were calculated by SPSS (Statistical Package for the Social Science). Dif-

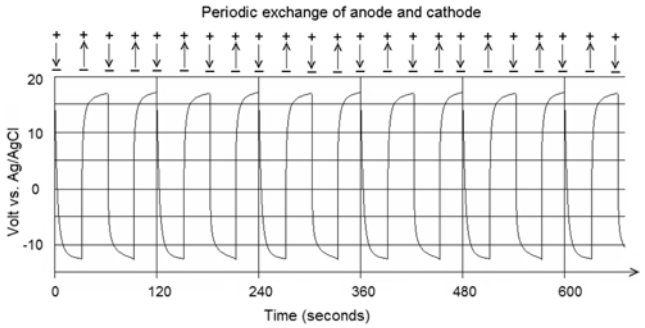

Fig. 2. Electric pulse generated by periodical exchange of electrode poles (anode and cathode), which was measured with Ag/AgCl reference electrode. The oxidation-reduction potential was swiftly changed to 17 volts at second converted from cathode to anode but changed to −10 volts at second converted from anode to cathode.

ference at $P<0.05$ was considered statistically significant.

RESULTS

1. ORP Variation in PEF

Oxidation-reduction potential (ORP) periodically exchanged in the PEF was measured in coupling with Ag/AgCl reference electrode (Fig. 1). The ORP reached to maximal 17 volts in anodic reaction and −13 volts in cathodic reaction. The 4 volt of ORP difference between anodic and cathodic reaction indicates that oxidation (anodic) reaction was more activated than reduction (cathodic) reaction (Fig. 2), by which oxidative current reached to 80 mA but reductive current reached to −40 mA (data not shown). The current variation in coupling with ORP variation is a clue that the periodically generated electric pulse may electrically oxidize or reduce some organic or inorganic compounds, by which soil weathering may be activated. The redox potential variation generated by anaerobic or aerobic bacterial metabolism can oxidize or reduce soil minerals, which induces some mineral ions to be dissolved or adsorbed [32].

2. Effect of PEF on Bacterial Growth

The ORP variation and oxidation-reduction reaction in the PEF may induce physicochemical variation of soil environment to be fluctuated. Mean viable cell number of the intrinsic bacteria in five different positions was very variable from 77,000 to 396,000 around electrodes and 339,000 to 680,000 around plants in the PEF (Fig. 3(a) and 3(b)), and 538,000 to 927,000 around plants in the conventional field (Fig. 3(c)). Bacterial number variation might be caused by both sampling time and sampling position difference according to the standard deviation variation. However, the *P*-value obtained based on five different positions was generally less than 0.05 (so that when statistical testing is performed, *P*-values of less than or equal to 0.05 are defined as indicating a statistically significant difference) [33]. Meanwhile, extrinsic bacteria (*R. solanacearum*) was relatively more inhibited than the intrinsic ones in the PEF based on the mean viable cell number from 15,000 to 47,000 around electrode and 152,000 to 374,000 around plant in the PEF (Fig. 4(a) and 4(b)), and 294,000 to 607,000 around plant in the conventional field (Fig. 4(c)). The *P*-value obtained based on the *R. solanacearum* viable cell number in five different positions was also generally less than 0.05.

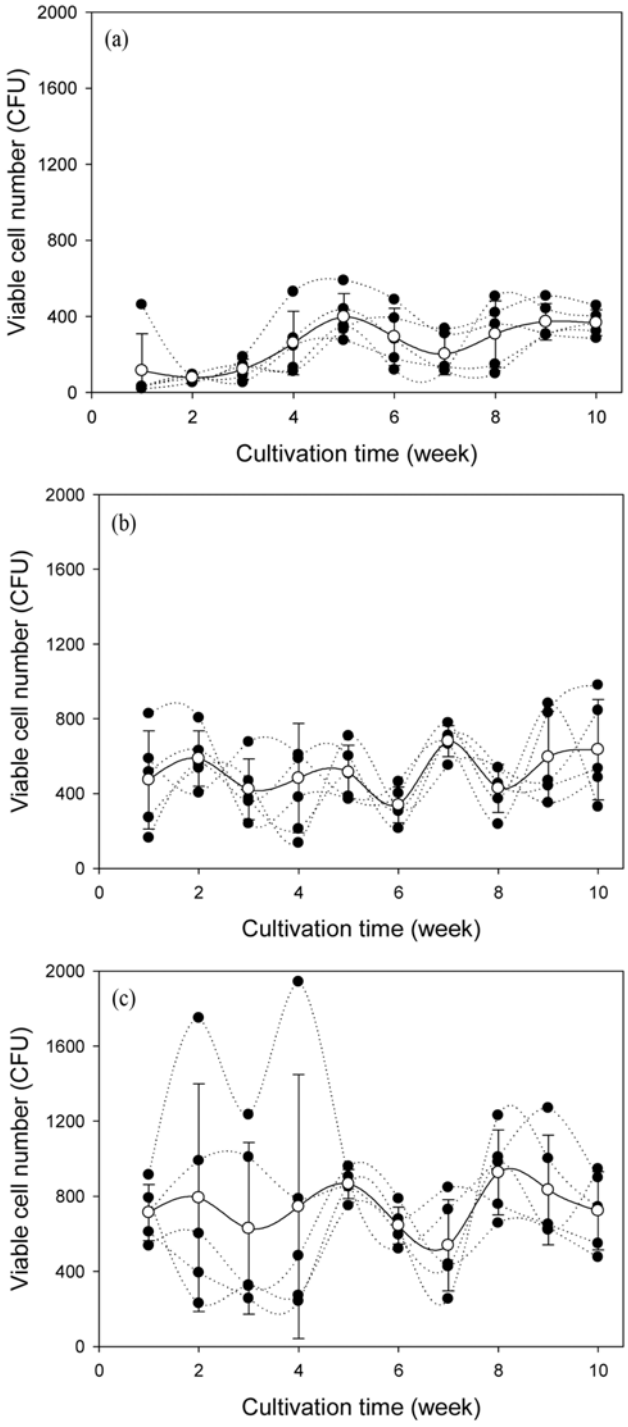

Fig. 3. Viable cell number (VCN) of the intrinsically growing bacteria in the pulsed electric field developed in hot pepper plant field and conventional field. Soils were sampled within 10 cm around electrode (a) and within 5 cm of hot pepper plant stem (b) in PEF, and within 5 cm of hot pepper plant stem in conventional field (c). Solid symbols (●) indicate the VCN variation in 5 different soil samples and open symbol (○) indicates mean VCN and standard deviation.

3. Effect of PEF on Bacterial Wilt

The hot pepper plants cultivated in flowerpots did not suffer from

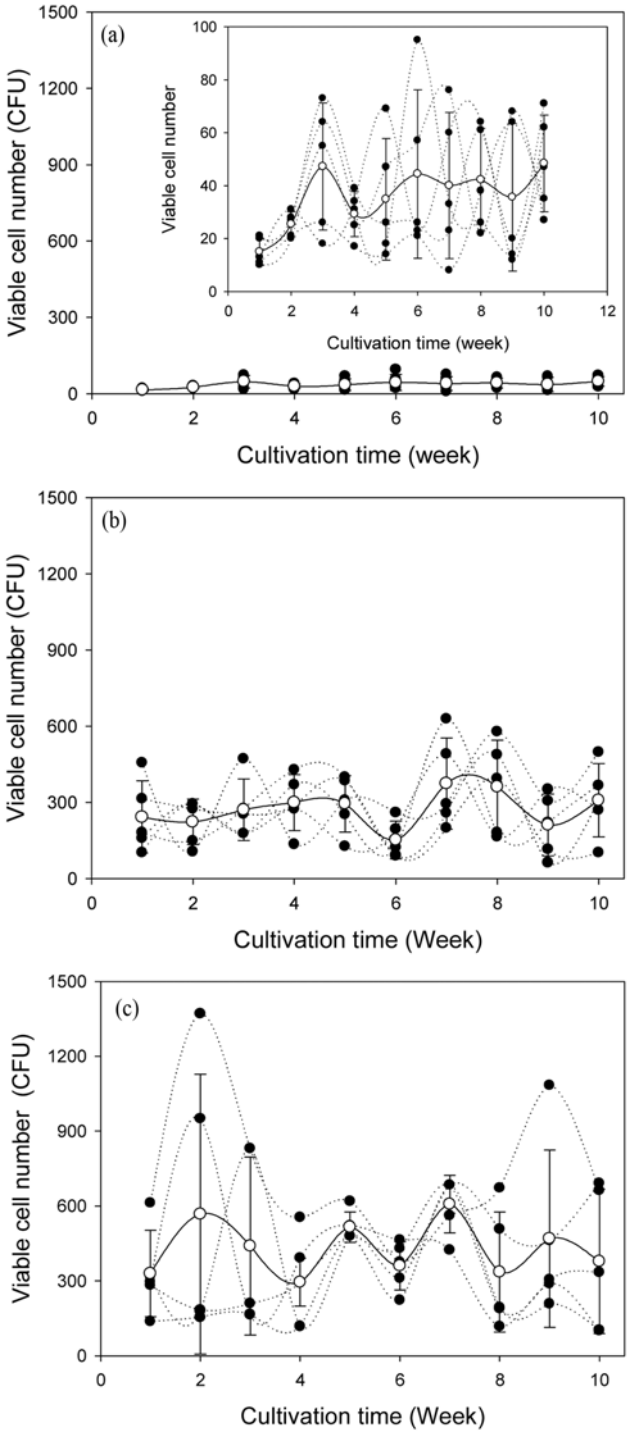

Fig. 4. Viable cell number (VCN) of the extrinsically contaminated bacterium *R. solanacearum* in the pulsed electric field developed in hot pepper plant field. Soils were sampled within 10 cm around electrode (a) and within 5 cm of hot pepper plant stem (b) in PEF, and within 5 cm of hot pepper plant stem in conventional field (c). Solid symbols (●) indicate the VCN variation in five different soil samples and open symbol (○) indicates mean VCN and standard deviation.

bacterial wilt for five weeks (data not shown); however, the transplanted hot pepper plants to the contaminated field with *R. solanacearum* by both passive and active infection suffered as shown in Table 1. Mean 3.93 and 5.67 of hot pepper plants were infected with bacterial wilt every two days by passive and active infection, respectively, in the conventional field. The suffering hot pepper plants from bacterial wilt were significantly decreased to mean 1.25 and 2.5 every two days by passive and active infection, respectively, in the PEF. Total hot pepper plants that suffered from bacterial wilt in the PEF were significantly lower than that in the conventional field. The fluctuating environment induced by PEF might reduce the population size of *R. solanacearum* (Fig. 4(a) and (b)). When the saps extracted from the infected plants with wilt disease were spread on plate count agar containing 0.005% of 2,3,5-triphenyl tetrazolium chloride, *R. solanacearum*-specific colonies were grown on an agar plate as shown in Fig. 5. The exopolysaccharide around bacterial colonies and red colored reaction are a typical property of *R. solanacearum*.

4. Effect of PEF on Plant Growth

The PEF significantly inhibited the growth of intrinsic soil bacteria and *R. solanacearum* but did not influence pepper plant growth. As shown in Table 2, the PEF did not inhibit the seed sprouting and hot pepper plant growth. Mean sprouting number of seeds in the PEF and conventional field was 45.0 and 48.2, respectively. Meanwhile, the mean dry weight of hot pepper plants was 3.15 g and 2.51 g in the PEF and conventional field, respectively. It is a clue that the environmental variation generated by PEF at 0.29-3 volt/cm of potential intensity would not be a noxious factor for hot pepper plants.

5. Effect of PEF on Bacterial Diversity

The bacterial diversity was estimated by comparison of the TGGE pattern for DNA extracted from five different sites in the PEF and conventional field at 5^{th} and 10^{th} week. As shown in Fig. 6, very minutely stacked lamella-like DNA bands on TGGE gel indicate that quite a variety of bacterial species are commonly living in both the PEF and conventional field. The TGGE band pattern in the PEF (A and C) was not very different in comparison with that in the conventional field (B and D) based on the band number, which corresponds to the bacterial diversity. None of DNAs extracted from TGGE gel was identified with *R. solanacearum*. All of the DNAs extracted from TGGE gel were identified with uncultured bacteria as follows: 1) Uncultured soil bacterium (94%, DQ054599), 2) Uncultured Crater Lake bacterium (94%, AF316788), 3) Uncultured bacterium (95%, EU883129), 4) Uncultured soil bacterium (94%, DQ642735), 5) Uncultured bacterium (97%, EU635959), 6) Uncultured bacterium (91%, FJ595574), 7) Uncultured soil bacterium (100%, DQ054625), 8) Uncultured soil bacterium clone (98%, DQ054625), 9) Uncultured soil bacterium (96%, DQ054609), 10) Uncultured bacterium (95%, DQ525809), 11) Uncultured soil bacterium (97%, DQ054629), 12) Uncultured bacterium (96%, EU776360), 13) Uncultured *Herbaspirillum* sp (90%, EU300333), 14) Uncultured bacterium (95%, FJ592574), 15) Uncultured bacterium (95%, FJ437486), 16) Uncultured bacterium (98%, AV487121), 17) Uncultured bacterium (97%, FM875632), 18) Uncultured bacterium gene for 16S rRNA partial sequence (95%, AB462557), 19) Uncultured bacterium (97%, FJ167453), and 20) Uncultured bacterium (98%, AB163874).

DISCUSSION

PEF induced from high electric voltage employed in the food

Table 1. The number of hot pepper plants passively (P) and actively (A) infected by *R. solanacearum* in conventional field (Conv) and pulsed electric field (PEF) during cultivation for 45 days after 150 young plants with 4-5 leaves were transplanted from non-contaminated flower pots

Days		Infected hot pepper plants with *R. solanacearum*															M	SD
		2	4	6	8	10	12	14	16	18	20	22	28	33	40	45		
Conv	P	3	0	0	6	7	5	7	6	7	5	2	3	3	4	1	3.93	2.46
		3	***3***	***3***	***9***	***16***	***21***	***28***	***34***	***41***	***46***	***48***	***51***	***54***	***58***	***59***		
	A	3	1	5	6	12	9	9	7	6	7	7	5	3	3	2	5.67	2.99
		3	***4***	***9***	***15***	***27***	***36***	***45***	***52***	***58***	***65***	***72***	***77***	***80***	***83***	***85***		
PEF	P	0	0	0	0	0	3	5	3	1	1	2	1	3	1	0	1.25	1.53
		0	***0***	***0***	***0***	***0***	***3***	***8***	***11***	***12***	***13***	***15***	***16***	***19***	***20***	***20***		
	A	0	0	0	4	4	5	6	3	1	2	3	2	4	5	1	2.50	2.03
		0	***0***	***0***	***4***	***8***	***13***	***19***	***22***	***23***	***25***	***28***	***30***	***34***	***39***	***40***		

M, Mean value; SD, standard deviation; SP (*P*-value) is commonly less than 0.001 (data not shown); Italic letters, cumulative number of infection plants

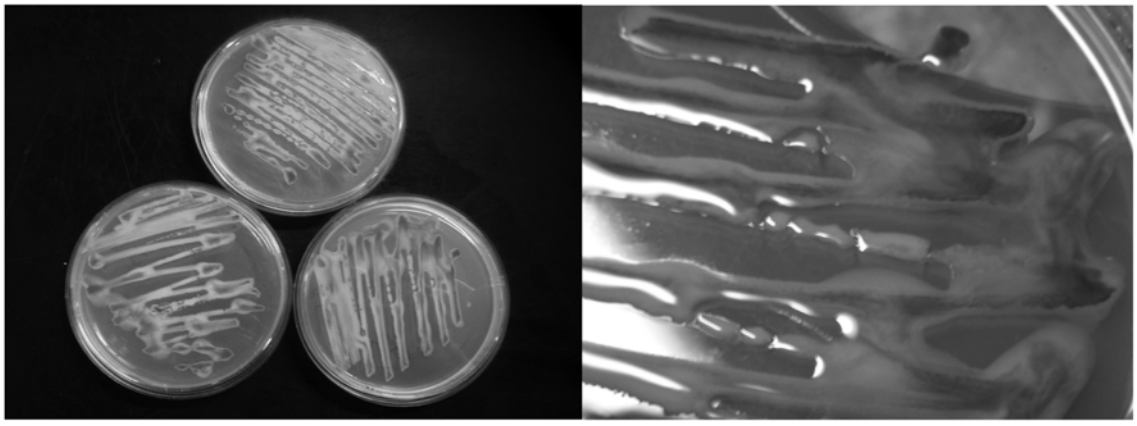

Fig. 5. *Ralstonia solanacearum* cells growing on agar plate, which was inoculated by streaking with cutting stem of hot pepper plants suffering from the bacterial wilt. The slime and color of bacterial cells grown on agar plate showed a typical feature of *Ralstonia solanacearum*.

Table 2. Numbers of sprouting pepper plants in the PEF and the conventional field after seeding, and mean dry weight of pepper plants after being cultivated for 50 days. Initially sowed seeds in each position were adjusted to 60

Conditions	Pulsed electric field							Conventional field						
Positions	1	2	3	4	5	M	SD	1	2	3	4	5	M	SD
Sprouting	49	47	49	48	48	48.2	0.83	45	45	46	45	44	45.0	0.71
Mean dry weight (g)	13	22	18	19	21	18.6	3.51	13	17	19	14	14	15.4	2.51

M, Mean value; SD, standard deviation; SP (*P*-value) was commonly less than 0.001 (data not shown)

process is not proper for controlling pathogenic bacteria growing in the rhizosphere because the strong electric shock may damage plant root cells [34,35]. Accordingly, an innoxious electric voltage for plant cells, but noxious for bacterial cells, has to be selected. In the previous test with a flowerpot culture, a few hot pepper plants slowly grew in 30 and 40 volts of the PEF; meanwhile, normal growth was observed under 10 and 20 volts of the PEF (data not shown). Therefore, it could be suggested that 20 volts of the PEF would be the maximal voltage incapable of interfering with hot pepper plant growth but the minimal voltage capable of inhibiting bacterial growth. The 20 volts of PEF employed in this research is greatly lower than that employed for bacterial inhibition in the food process [36,37]. One research suggested that the application of high intensity PEF (20 kV/cm) to liquids can inactivate susceptible microorganisms through irreversible electroporation of the cell membrane [38]. The electroporation activity of the high intensity PEF may be a cause to damage plant cells; however, about 0.3 volt/cm of electric intensity destroyed neither bacterial cells nor plant cells, which is too low to induce the electroporation of bacterial membrane and plant cell membrane. However, the bacterial cell number was meaningfully decreased in the PEF, and the sprouting and growth of hot pepper plants was not influenced by the PEF generated from 0.3 volt/cm of electric intensity. It is very possible that 0.3 volt/cm of electric intensity might not be a factor to damage bacterial cells but may induce the

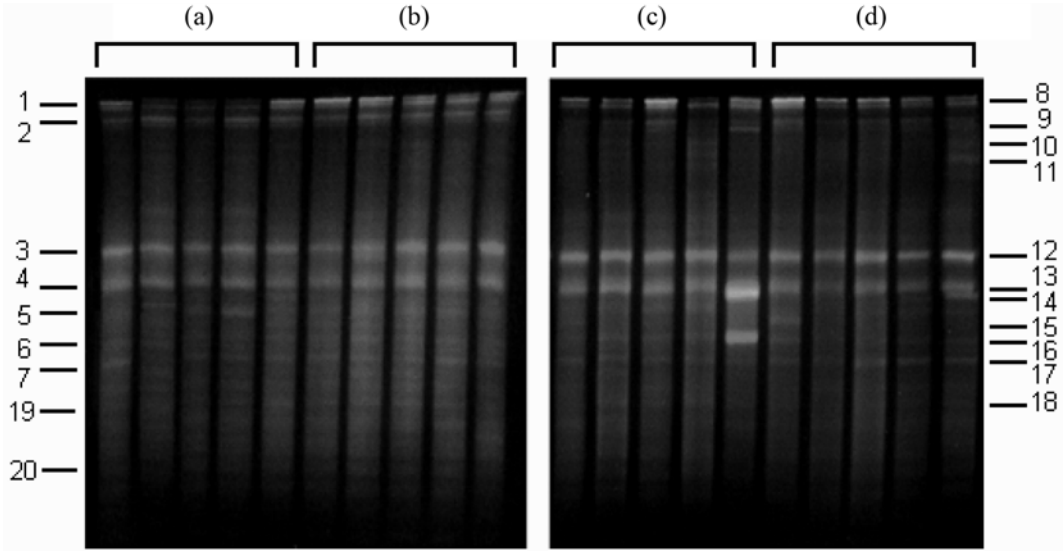

Fig. 6. TGGE pattern of bacterial DNA extracted from soils in five different positions of the PEF (a) and conventional field (b) at fifth week and those of the PEF (c) and conventional field (d) at tenth week.

environments of bacterial habitats to fluctuate [39]. The continuously changing environment may induce bacterial cells to consume more physiological energy than a stable environment. In a natural environment, the competition power of bacterial cells for growth and survival is proportional to the metabolic energy production [40]. The PEF is possible to induce bacterial cells to consume more maintenance energy required to adapt to the fluctuating environment, by which the bacterial regeneration may be retarded [41]. The PEF effect capable of decreasing the bacterial number but maintaining bacterial diversity is very useful because the bacterial ecosystem in any crop field has to be preserved. Various root-associated bacterial communities can contribute to the mineral weathering and mineral nutrition for plants [42]. The mineral weathering by soil microorganisms has been known to be required for ion cycling and plant nutrition [43-45]. The microorganisms capable of weathering minerals belonged to the α-proteobacteria (*Agrobacterium* and *Rhizobium*), β-proteobacteria (*Achromobacter* and *Burkholderia*), the γ-proteobacteria (*Acidithiobacillus*, *Aerobacter*, *Citrobacter*, *Enterobacter*, *Erwinia* and *Pseudomonas*) and gram-positive bacteria (*Bacillus* and *Micrococcus*) [46]; however, all of the partial 16S-rDNA extracted from TGGE bands was identified as uncultured soil or uncultured bacterium. So many bacterial DNA sequences are released in the GenBank database system that a specific bacterial species is accurately identified. Various techniques such as biological control, chemical control, cultural control and host resistance have been studied and applied to food crops to protect or prevent bacterial wilt [47]. Recently, integrated control strategies using various techniques at the same time, for example, cultivation of wilt-tolerant crops, short rotational cultivation, intercropping, control of planting data, soil amendment, and cultivation in *R. solanacearum*-free soil have been designed and employed in the field [48]. However, a technique for the complete protection from bacterial wilt disease has not been developed because the pathogens that cause wilt disease are infected from plant roots. The PEF may be one of the techniques applicable to integrated control strategies to protect and prevent bacterial wilt.

CONCLUSION

The low intensity of PEF developed around roots of pepper plants is supposed to be an environmental factor. In the statistical and genetic analysis of bacterial communities in the pepper plant field, the PEF did not destroy bacterial diversity but induced the bacterial community size to decrease. The hot pepper plant growth was not influenced by the PEF. We supposed that the PEF may induce the biogeochemical condition of the bacterial environment to continuously fluctuate or change; however, the mechanism on how the PEF can control bacterial growth is required to be biochemically and electrochemically examined. The work for *in situ* evaluating the PEF effect on bacterial growth by using a hydroponic culture technique is proceeding.

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