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Pretreatment with an esterase from the yeast *Pseudozyma antarctica* accelerates biodegradation of plastic mulch film in soil under laboratory conditions

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The yeast *Pseudozyma antarctica* secretes a concentrated biodegradable plastic (BP)-degrading enzyme when cultivated with xylose. Treatment with the culture filtrate reduced the puncture strength of commercial BP mulch films. After burying the film in soil, the residual amount of solid film was reduced significantly, and none was recovered after 5 weeks. The dynamics of soil fungal communities were analyzed weekly after burying the film using 18S rDNA polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiling of soil DNA. In the soil containing enzyme-treated film, the native community essentially recovered within 24 weeks. In comparison, the untreated solid film remained in the soil for 12 weeks and the response of the soil-fungal community was relatively slow; it had not recovered within 24 weeks.

[**Key words:** *Pseudozyma antarctica*; Esterase; Biodegradable plastic; Polymerase chain reaction-denaturing gradient gel electrophoresis; Soil-fungal community]

Agricultural plastic mulch films are used to cover soil beds for time periods of two to six months for weed control and maintenance of soil temperature and moisture for the stable production of high-quality vegetables (1). The market for low-cost, durable polyethylene (PE) mulch is growing rapidly (2), and about 1.8 million tons were consumed worldwide in 2015 (3). Since the PE mulch film is stable in soil, it is necessary to recover and dispose of the used film as an industrial waste. However, this process is labor-intensive, and disposal and recycling of the soil-contaminated plastic film is difficult. Additionally, higher relative proportions of unrecovered film remain in the field when the mulch film is thinner, and large amounts of film accumulate in soil over time (1,4,5).

One solution to these problems is to use biodegradable plastic (BP) mulch films. BP mulch films can provide the proper strength during cultivation, but degrade rapidly after harvest. The ideal BP mulch films retain properties similar to those of PE initially and then degrade gradually, beginning with fragmentation or surface erosion, with a decrease in performance properties at the latter part of the cultivation period. At the end of the growing season, the films can be tilled into the soil, where they would undergo further chemical change. The chemical change must be followed by complete microbial assimilation of degradation products, resulting in CO_2, water, and cell biomass, without any long-term accumulation of plastic. Since there is no need to remove the mulch films from the field, the use of such BP mulch films provides benefits to farmers and reduce the environmental impact of plastic used in cultivation (6).

The rate of mulch degradation depends on the chemical composition of the polymer (6). The strength and degradation speed of BP mulch film is controlled by the blending ratio of aliphatic and aliphatic-aromatic co-polyester compounds of various biodegradabilities, such as polybutylene succinate-*co*-adipate (PBSA), polybutylene succinate (PBS), and polybutylene adipate-*co*-terephthalate (PBAT) (3). However, the degradation speed of polymers in the field soils is variable because it is also affected by a range of agro-climate conditions, such as humidity, temperature, soil properties, and microbial activity (7). Under the influence of these environmental factors, the polymers are hydrolyzed to low molecular mass water-soluble oligomers and finally produce monomers that are assimilable by various soil microorganisms as carbon sources. Thus, we suspect that BP films change the soil microorganism community as long as they remain in the soil. A few studies have examined the breakdown of buried BP mulch films in soil. The soil degradability of three commercially available mulch films after tomato cropping seasons at five locations in Spain varied between 25% and 88%, and the degradability was dependent on the mulch type used and the cropping location (7). Although BP mulch films degrade gradually in soil, the fragmented mulch films sometimes remain in the soil longer than farmers expect and may appear on the soil surface.

Kyrikou and Briassoulis mentioned that a technique for the induction of severe degradation at the desired timing would be useful to avoid accumulation of BP films in soil (6). With this background, we attempted to develop a technique for accelerating the degradation of commercial BP mulch films by treating them with BP-degrading enzymes before plowing (8). We previously spray-treated a BP-degrading enzyme of the fungus *Paraphoma* sp. strain B47-9 (PCLE) onto BP mulch films in agricultural settings and demonstrated visible breakdown of the films one day

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after treatment (8). The yeast *Pseudozyma antarctica*, which was isolated from rice leaves and husks, secretes an esterase (PaE) that can degrade PBS, PBSA, and other types of BP films (9–11). We obtained a filtrate of *P. antarctica* strains cultured with xylose that contained highly concentrated PaE (11). Cut pieces (1 × 1 cm, 20-μm thick) of commercial BP mulch films made of PBS, PBSA, and PBAT lost their original form after being submerged in the culture supernatant for several days (11). PaE randomly degrades the polyester polymers into monomer units (endo-type degradation) faster than PCLE does (12), so we considered PaE to be a promising candidate for accelerating the degradation of used BP mulch films in the field. For the practical use of this technique, it is necessary to clarify the effects of PaE pretreatment on the decomposition speed of BP films in soil and on the soil microorganism community.

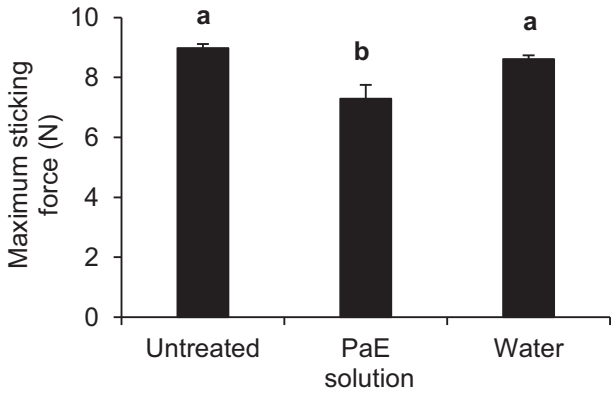

FIG. 1. The effect of PaE solution treatment on film strength. Each of four films that were untreated or treated with PaE solution or water was penetrated five times with a digital force gauge. The data are the averages of the 20 points and bars indicate the standard error of the mean. The same letters above two columns indicate that the values were not significantly different according to the Tukey test ($P < 0.05$).

In this study, we treated commercial BP mulch films with the culture filtrate (PaE solution). After burying samples of the film in farm soil, the degradation of the films and microbial transition in the soil were monitored under laboratory conditions.

MATERIALS AND METHODS

Materials Commercial 20-μm-thick black BP mulch film made of PBS, PBSA, and PBAT in a butanediol monomer-based molar ratio of 49:37:14 (13) was studied.

P. antarctica (*Moesziomyces antarcticus*) GB-4(1)W was used for preparation of culture filtrate containing PaE. The culture filtrate of *P. antarctica* GB-4(1)W was prepared as described previously (11), and used as the PaE solution. The enzyme activity of the PaE solution was evaluated based on the decrease in the turbidity of emulsified PBSA, as described previously (9). One unit of PBSA degradation activity was defined as a 1 OD_{660} decrease per min in the reaction mixture (20 mM Tris-HCl, pH 6.8). The activity of the PaE solution was 4.2 U.

Soil (Andisol; total carbon 5.67%, total nitrogen 0.46%, measured by NC analyzer Sumigraph NC-22F [Sumika Chemical Analysis Service, Osaka, Japan] according to the manufacturer's instructions) was collected from a depth of 10 cm in a greenhouse at the Institute for Agro-Environmental Sciences, NARO, Japan (36°01′31.3″ N 140°06′19.0″ E). The soil was passed through a 2-mm sieve, air dried for several days, and stored at 4 °C until use. At the beginning of the experiment, the moisture content of the soil was adjusted to 50% of the maximum water-holding capacity, and the soil was kept at 25 °C.

Treatment of BP films with PaE solution Each cut BP film was exposed to PaE solution (40 μL/cm^2) on both sides in a stain-less steel tray, and dried naturally for 18–24 h at room temperature. Films were treated similarly with control solutions (distilled water and autoclaved PaE solution after confirming that the BP-degrading activity of the PaE solution was inactivated). After the treatment, the films were rinsed with distilled water, and dried on paper towels for 24 h.

Evaluation of film strength with a sticking test Four pieces of cut film (5 × 15 cm) treated with PaE solution or distilled water were evaluated. The maximum sticking force required for penetrating the film was measured at five random points in each film using a digital force gauge (ZP-50N; Imada, Aichi, Japan) at a velocity of 4 mm/s.

Evaluation of the BP film degradation in soil Soil (130 g) was placed in a sealed 400-mL container (TiteBox, inside dimensions 127 × 87 × 41.5 mm; AsOne, Osaka, Japan). Six 2 × 2-cm pieces of BP film were treated with PaE solution for 18–24 h, as described above, and packed in the soil at regular intervals. Similarly, six pieces each of film treated with autoclaved-PaE solution and sterilized water were buried in soil in each container. Five containers were prepared for each short treatment (1–6 weeks) and incubated at 25 °C. To maintain the soil moisture, the decrease in the weight of

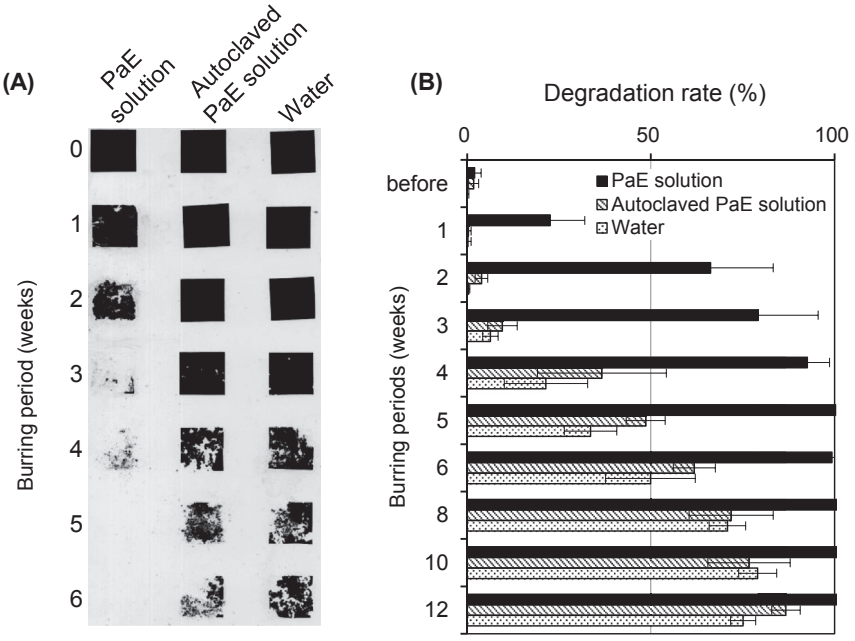

FIG. 2. Effect of PaE solution pretreatment on the degradation of commercial BP films in soil. (A) Images of the residual of each treated BP film after being buried for 6 weeks. (B) Comparison of the degradation rates of each treated BP film after being buried for 12 weeks. Closed columns, PaE solution-treated films; cross-hatched columns, autoclaved PaE solution-treated films; dotted columns, water-treated films. Each value represents the average of five determinations and each error bar indicates the standard deviation of the mean.

each container was measured, and an equal weight of distilled water was sprayed on the soil surface every 4–7 days. Five containers for each treatment were also prepared for longer incubations (8, 10, and 12 weeks), as described above.

One piece of film was collected from each container every week for 6 weeks, and at 8, 10, and 12 weeks. Films were gently rinsed by water. After removing the soil, the collected films were sandwiched between transparent laminating films, and the modulation of the luminance intensity of the 2 × 2 cm area around the film was measured, as described previously (9). The degradation ratio (%) was calculated as:

$$\text{Degradation ratio}\,(\%) = \frac{(\text{Luminance of residual film}) - (\text{Luminance of fresh film})}{(\text{Luminance of background}) - (\text{Luminance of fresh film})} \times 100 \quad (1)$$

Observation of BP film degradation by scanning electron microscopy To observe the morphology of the film surface, the film was sputter-coated with platinum particles using a sputter coater (model E-1010; Hitachi, Tokyo, Japan) and was observed under a scanning electron microscope (SEM, model JSM-5610LV; JEOL, Tokyo, Japan) with an accelerating voltage of 20 kV. The resulting images were captured digitally.

PCR-DGGE analysis of fungal 18S rDNA fragments in BP film-degrading soil Six pieces of BP film (1 × 1 cm) treated with PaE solution or sterilized water were buried at regular intervals in soil in sealed containers, as described above. As a control, sealed containers were also prepared with soil, but no film. Two containers were prepared for each treatment, and incubated at 25 °C at a constant moisture content. Two more containers were also prepared for each longer (4–24 weeks) incubation. A soil sample (approximately 2 g from near one piece of film) was collected from each container every week for 6 weeks or every 4 weeks for 24 weeks. The collected soil samples were stored at −30 °C.

The soil samples were ground homogeneously and DNA was extracted from a 0.4-g soil sample using a FastDNA Spin kit for soil (Qbiogene/MP Biomedicals, Solon, OH, USA), according to the methods described by Hoshino and Matsumoto (14). Bacterial 16S rDNA and fungal 18S rRNA genes from soil DNA were analyzed as described by Tsushima and Matsushita (15). Sequencing of DGGE bands was performed as described by Hoshino and Matsumoto (14), and PCR amplification to recover the 18S rRNA gene was done with primer pair NS1 and GCFung under the original PCR conditions.

Analysis of the DGGE profiles DGGE profiles were digitized using GelCompar II software (Applied Maths, Austin, TX, USA). Principal component analysis (PCA) of the PCR-DGGE profiles was performed to elucidate the fungal community structure based on the relative band intensity and positions using R ver. 2.15.0 software (16) (https://www.r-project.org/).

RESULTS

Effect of PaE solution treatment on film strength After the PaE solution treatment, the average puncture resistance of five random points in each of four cut films (7.29 N, SE = 0.47) was reduced significantly compared with that of non-treated films (8.98 N, SE = 0.14). The puncture resistance of the water-treated film (8.61 N, SE = 0.13) was the same as that of the untreated film (Fig. 1).

Effect of PaE solution pretreatment on film degradation speed in soil After treatment with PaE solution, the glossy surface of the films changed to a matte surface. These films treated with PaE solution were then placed in soil. After a 1-week incubation period, the excavated films were surrounded by soil flocs and fungal hyphae. The films degraded gradually, and solid fragments of film could not be recovered from the soil after a 5-week incubation (Fig. 2A). For comparison, films treated with distilled water and autoclaved PaE solution were buried in soil as controls. After a 2-week incubation in soil, the excavated control films were not surrounded by soil flocs and separated from soil easily. After a 4-week incubation, both excavated control films were surrounded by soil flocs and hyphae, and the residual film was visibly broken (Fig. 2A). The films persisted in the soil after a 5-week incubation, but 14–25% of the films could be collected after a 12-week incubation (Fig. 2B).

Observation of BP film degradation by SEM The films were observed by SEM. The surface of the water-treated film was smooth, but the PaE solution-treated film was rough and many holes had formed in it (Fig. 3A). After a 2-week incubation in soil, significant erosion and many larger holes were observed on the surface of the PaE solution-treated film beneath soil granules and fungal hyphae (Fig. 3B, c and d). The degradation proceeded along one direction of the film because the amorphous region was etched faster than the crystalline region (17). On the other hand, some crevices were observed on the smooth surface of the control film after a 2-week incubation (Fig. 3B, a and b).

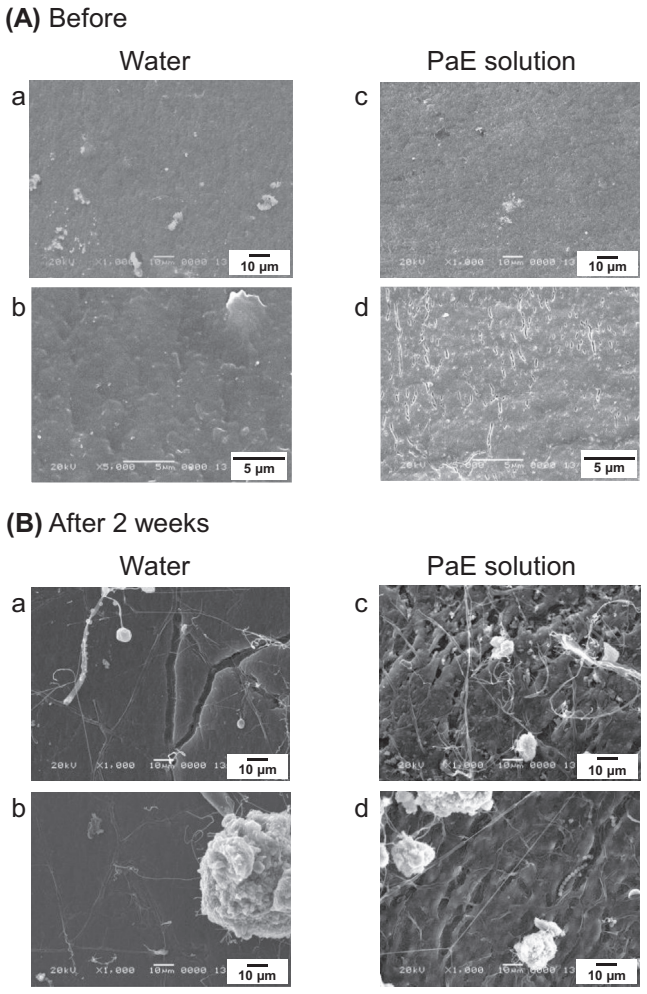

FIG. 3. SEM micrographs of film surfaces of (A) water-treated films (a, b) and PaE solution-treated films (c, d) before incubation and (B) water-treated films (a, b) and PaE solution-treated films (c, d) after incubation in soil for 2 weeks. The scale bars in panels a and c in Fig. 3A, and 3B indicate 10 μm. The scale bars in panels b and d in Fig. 3A indicate 5 μm.

DGGE profiles of the microbial community after the films were buried in soil To elucidate the influence of PaE solution-treated BP mulch films on the soil microorganisms, soil samples in containers were incubated with films after PaE solution treatment, films after water treatment, and with no film. The 16S rDNA gene fragments of bacteria and the 18S rDNA gene fragments of fungi amplified by PCR from the total community DNA of soil samples from the longer-incubation-time containers (4–24 weeks) were analyzed by DGGE. No significant changes were observed in the PCR-DGGE profile of the 16S rDNA gene fragment (Fig. S1), indicating that the inoculation of the film and the PaE solution-treated film did not significantly influence the bacterial communities in the soil. On the other hand, there was obvious change in the fungal community in the soil samples with the BP films (Fig. 4), as described below.

To analyze the changes in the soil fungal community at earlier stages of incubation, we analyzed 18S rDNA isolated from soils with shorter incubation times (0–6 weeks) by PCR-DGGE. Photographs

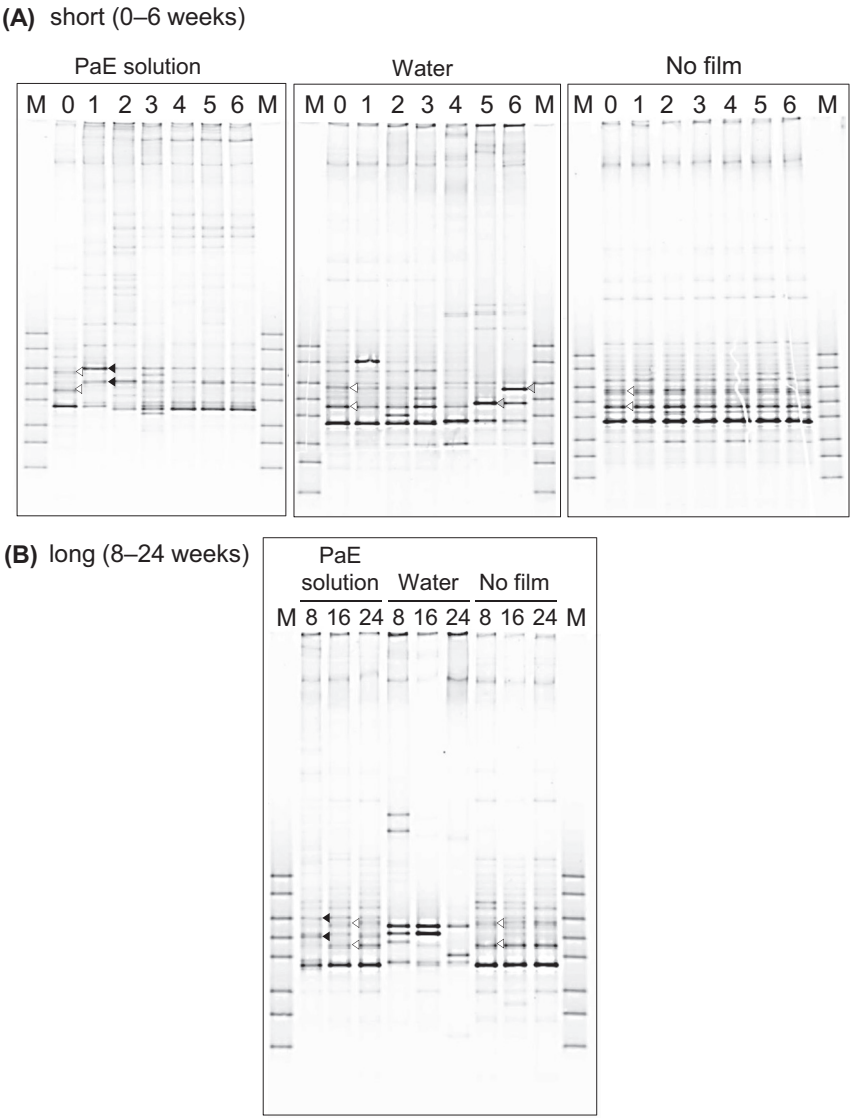

FIG. 4. DGGE profiles of the 18S rDNA-based fungal community in soil containing PaE solution-treated films, water-treated films, and no film for (A) short (0–6 weeks) and (B) long (8–24 weeks) incubations. M: DGGE marker. Lane numbers indicate the number of weeks of incubation. White arrows indicate the major bands that were observed in soil samples before incubation or with no film. Black and gray arrows indicate the major bands observed in film-containing soil.

of PCR-DGGE gels of samples corresponding to shorter incubation times (0–6 weeks) and longer incubation times (8–24 weeks) for container 1 of the two tested containers used for each treatment are presented in Fig. 4. In the soil containing the PaE solution-treated film, changes in the PCR-DGGE profile were observed at an early stage of the incubation. Two new major bands (black arrows) appeared after 1 week. Bands with the same mobility (Rf value) were also detected in 2–6-week (Fig. 4A) and 8-week (Fig. 4B) soil samples. Additionally, two major bands observed in the original soil (white arrows) were not visible after 1 week of incubation (Fig. 4A), indicating that the relative amounts of the DNA decreased in the soil with the PaE solution-treated film. However, bands with the same Rf values as those shown by the white arrows were detected in the samples from 16 to 24 weeks of incubation (Fig. 4B). On the other hand, for the soil containing the water-treated film, the two bands with the same Rf values as the white arrows were detected until 3 weeks of incubation. Changes in the major bands were observed after 4 weeks of incubation, and new major bands (gray arrows) appeared at later stages of incubation. The PCR-DGGE profile was stable in the soil that did not contain any film, and the two bands with the same Rf value as the white arrows were detected during the entire incubation period.

The community structures obtained from the DGGE profiles were analyzed by PCA (Fig. 5). The PCA plot profiles explained 45.5% of the variance in the fungal community. The structure of the original fungal community was present at week 0 in every treatment and in the soil without film. An immediate response was observed within 1 week for the soil that contained PaE-treated film (Figs. 4A and 5A). The DGGE profiles of the soil with enzyme-treated film and the soil without film were similar after 16–24 weeks (Fig. 4B). The original fungal community structure of the soil with PaE solution-treated film began to recover at 8 weeks of incubation (Fig. 5A). The recovery was almost complete within 24 weeks. In comparison, in the soil with the water-treated film, the fungal community structure began to change after 4 weeks of incubation, but did not recover within 24 weeks (Figs. 4A, B and 5B).

Analysis of PCR-DGGE gel-separated 18S rDNA gene fragments from the soil samples after longer incubation times (8–24 weeks) showed several major bands which were extracted and their DNA sequences were analyzed (Fig. S2). Based on comparison with the

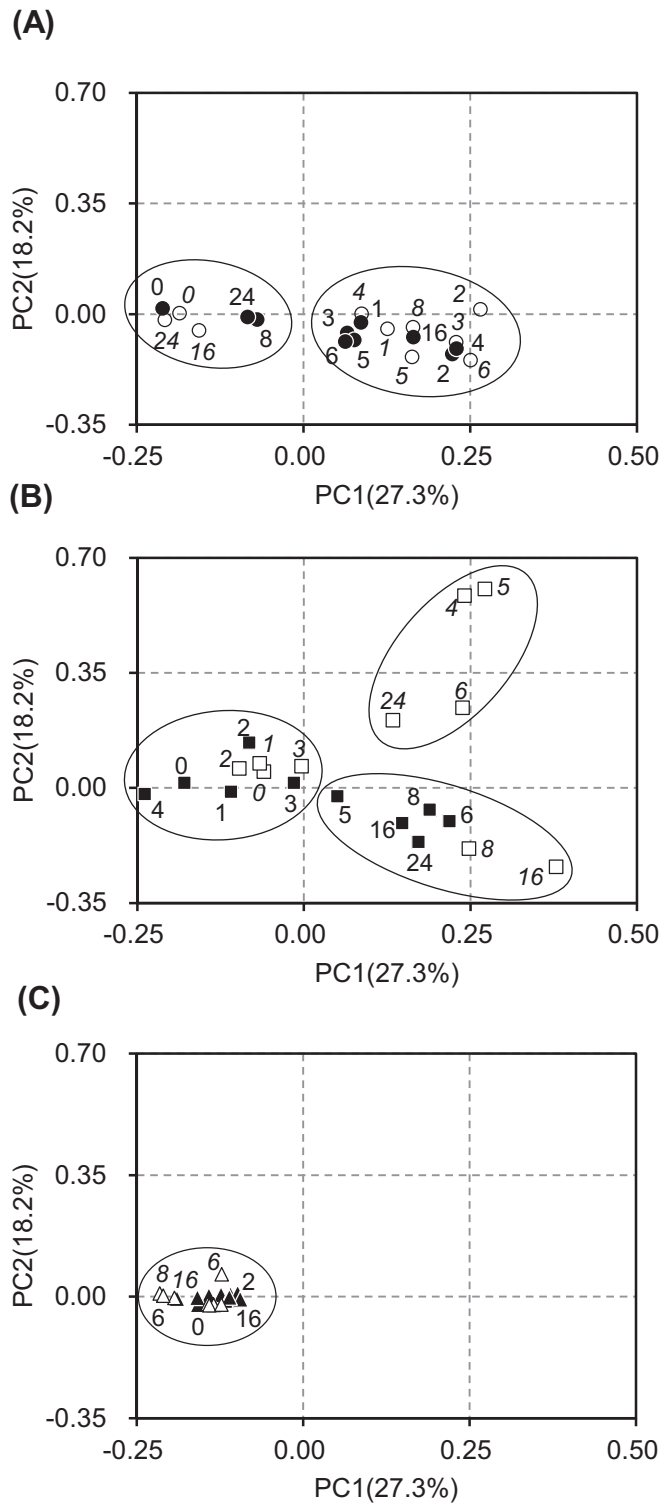

FIG. 5. PCA analysis of the DGGE profiles of the 18S rDNA-based fungal community in soil harboring BP films for 0–24 weeks. DNA samples of soils containing PaE solution-treated films (A), water-treated films (B), and no film (C). Numbers indicate the number of weeks of incubation. Closed and open symbols indicate two independent experiments.

DNA sequences in GenBank, one species shared the highest percent identity among the hits, as listed in Table S1. Fungi related to *Chaetomium globosum* and *Actinomucor elegans*, known to be saprobic fungi, were detected in the original soil and in the soil containing films. In the soil containing films, fungi highly related to *Spiromyces minutus*, *Aspergillus ustus*, *Fusarium* sp., and *Seltsamia ulmi* were detected. However, these bands were faint or not detected in the original soil. A more detailed analysis of the microorganism community transition during the film decomposition process could be performed by metagenomic analysis using a next generation sequencer.

DISCUSSION

In this study, based on puncture resistance analysis, we observed that the PaE solution treatment decreased the mechanical strength of the BP mulch films (Fig. 1). Films treated with PaE solution exhibited markedly greater degradation speed in soil than films treated with water (Figs. 2 and 3). The soil fungal community was disturbed after film inoculation, but community recovery was observed after 24 weeks in the soil containing PaE solution-treated films (Figs. 4 and 5). These changes were significantly slower in the soil with water-treated film, and recovery of the fungal community was not observed within 24 weeks of incubation.

In the soil containing buried PaE solution-pretreated films, the fungal community was disturbed more substantially by the degrading film than the bacterial community. This result coincides with reports that showed significant change in the soil fungi community in soils incubated with PBSA (18), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (19), or PBAT (20) films. Visible degradation of the film (Fig. 2) and an immediate response in the fungal community was observed after 1 week in the soil that contained PaE-treated films (Figs. 4A and 5A). The original fungal community structure of the soil with enzyme-treated films began to recover at 8 weeks of incubation, and the recovery was almost complete within 24 weeks based on the DGGE profile analysis (Fig. 5A). Although we do not have direct evidence showing that the soil microorganisms contributed to the degradation and assimilation of the films in soil, the results indicated that biodegradable and assimilable carbon derived from the buried film was consumed in the soil by soil microorganisms.

Films treated with distilled water were buried in soil as controls. After 2 weeks of incubation, the films were visibly stable, but some crevices were observed on the film surface by SEM (Fig. 3B, a and b). The degradation of the films was progressive. Films had visible holes after 3 weeks of incubation and were fragmented but were still present in the soil after 6 weeks of incubation (Fig. 2). The fungal community structure began to change after 4 weeks of incubation and did not recover within 24 weeks (Figs. 4A, B and 5B). Thus, it took a longer time for the film to degrade, and residual carbohydrates derived from films remained in the soil.

The PaE solution contained nutrients derived from the medium. To distinguish between the effects of enzyme activity and the nutrients in the PaE solution on the degradation of the film, films treated with autoclaved PaE solution were buried in soil. Because the degradation speed was the same as that observed for the water-treated films, the residual nutrients in the culture filtrate did not influence the soil microbe activity or the film degradation.

In the previous report on soil containing buried PHBV, the population of fungi capable of degrading PHBV particles increased (19). Moreover, in the soil containing buried PBSA film, the esterase activities and viable counts of PBSA-degrading fungi increased with time. However, PBSA film degradation in sterilized soil was very slow compared to the degradation in unsterilized soil. Therefore, the biodegradation of BP polymers is considered to depend on the action of fungi in soil, and these fungi accumulate in soil containing BPs (18). In the soil containing buried PBAT film, the fungal population increased relative to the bacterial population and, based on the PCR-DGGE analysis, the fungal community changed significantly (20); however, they did not determine the degradation ability of the fungi

that propagated. Commercially available BP mulch films are composed of several biodegradable polymers with relatively slow degradability (21). The effects of the BP mulch films on the soil microbial community and the contribution of these microorganisms to the degradation of BP mulch films are not well studied. It has been stated that aliphatic polyesters can be degraded nonenzymatically in the environment via simple chemical hydrolysis (17). Nevertheless, the chemical structures of polymers in soil change during incubation (22). However, soil fungi isolated from commercial BP mulch film buried in field soil for 6 months were not able to substantially degrade fresh BP mulch films in the laboratory on agarose plates (23). These results indicate that both abiotic reactions and the activity of microorganisms are involved in hydrolyzing polymers and oligomers in soil (6). On the other hand, we previously observed that fresh commercial BP mulch film (the same film used in this study) lost its original form after being submerged in PaE solution (11). For PBS and PBSA films treated with PaE solution, it was shown that the average molecular weight of the residual films decreases with reaction time (12). As the reaction proceeds, the smaller molecular weight water-soluble oligomers and monomers released by PaE are solubilized into the reaction solution (12). Consequently, more soil microorganisms can obtain degradable, assimilable carbon from the surface of the PaE solution-treated films compared to the case of water-treated films. Thus, rapid change in the soil fungal community was observed in the soil containing PaE solution-treated films based on the PCR-DGGE analysis (Figs. 4A and 5A).

In conclusion, we demonstrated that pretreatment with a BP-degrading enzyme accelerated the speed of commercial BP film degradation in soil. The amount of residual solid fragments in soil was decreased, and no solid fragments were recovered from soil after 5 weeks. Therefore, enzyme pretreatment decreased the accumulation of used BP mulch films in soil. The soil fungal community changed with film inoculation, and the change continued after the degradation of solid fragments of film. In soil containing buried BP films, some of the accumulated microorganisms may contribute to the degradation process of BPs. Accordingly, it is expected that the degradation speed of BP films would increase in soil that had accumulated BP-degrading microorganisms. To use BP mulch films of the same stability in a subsequent cultivation, it is important to reset the BP-degrading potential of farm soil. The enzyme pretreatment of BP mulch films reduced the period required for recovery of the original soil fungal community. Therefore, the combined use of BP mulch films and BP-degrading enzymes is a useful technique for continuous use of BP mulch films, which decreases the environmental impact of BP mulch film use and contributes to agroecosystem sustainability.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jbiosc.2018.06.011.

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