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Mechanism of Fungal Degradation on Military Aircraft Coatings

SERDP Project WP-2746

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Irfan S Ahmad
Hanafy M. Fouly
Rohit Bhargava
Taher Saif
Rajveer Singh
BOT University of Illinois

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Drs. Irfan S. Ahmad, Hanafy M. Fouly, Rohit Bhargava, Taher Saif, and Rajveer Singh

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ABSTRACT

Objectives: Project WP-2746, "Mechanism of Fungal Degradation of Military Aircraft Coatings" was undertaken using advanced instrumentation techniques and methods for conducting an exploratory investigation on chemical degradation and mechanical penetration of coatings to reduce maintenance costs.

Technical Approach: Degradation of aircraft coating systems is of vital importance from a safety standpoint as well as aesthetically, and results in significant maintenance cost to the aircraft industry. Early detection of degradation is critical to prevent complete failure and delamination. Development of methods based on spectroscopic techniques are required because of their potential to be used for *in-situ* assessment. Aircraft coating systems, usually containing polyurethane, are prone to degradation through microbes and harsh weathering conditions. Our broader goals in this project were three folds: (1) isolate, optimize fungal growth, and identify various potential fungal species from degraded coats, (2) investigate physical penetration of isolated fungi using Atomic Force Microscopy (AFM), and Environmental Scanning Electron Microscopy (ESEM) and (3) assess chemical degradation using Raman and FTIR spectroscopy techniques. In this project report, fungal penetration and chemical degradation of in-house and US Army prepared model coating systems has been reported. We first optimized growth conditions for the two fungal species, *Aureobasidium pullulans,* and *Fusarium spp.* on Potato Dextrose Agar medium. Second, model coating systems were prepared on Al-coated glass slides with (A) white Universal bonding primer (Rust Oleum®) coating and (B) white Universal bonding primer + a clear top coat (Dupli-Color®). Third, fungi and the two coating systems were characterized using Attenuated Total Reflectance- Fourier Transform Infrared (ATR-FTIR) spectroscopy to obtain corresponding reference spectra. An accelerated fungal-biodegradation study was also conducted by inoculating the two coating systems with pre-grown fungi isolates at 28^0C and 80% humidity for ~8 weeks.

Results: The AFM and confocal laser scanning microscope scans showed the tested fungal isolates grew well, penetrated, and produced chemical and topological changes to the model coating system. In addition, the ATR-FTIR analysis of the fungal exposed samples showed a decrease in the bands' intensities corresponding to various polyurethane bands (1726 cm^{-1} Carbonyl (C=O) species;1466 cm^{-1} Aliphatic (CH_2) stretch polyurethane backbone; 1240 cm^{-1} (C=O) + (O-CH_2) stretch; 1147 cm^{-1} C-O-C stretch). Control coating systems were similarly incubated without being exposed to the fungi, so they did not show any fungal degradation. Fungal exposed samples showed an increase in Amide-I band (1653 cm^{-1}) intensity indicating biomass growth. At a later stage in the project, a spore suspension of each of *A. pullulans* and a consortium of fungi were used to inoculate US Army coating systems. Data from ESEM showed fungal physical penetration, cracking and damage to US Army coating systems. It was intriguing to observe that the clean samples of US Army paint borne coating systems were contaminated with several mold fungi such as *Penicillium, Aspergillus, Phycomycetes,* and other unidentified isolates of fungi.

Benefits: Further work on the project is likely to result in improved maintenance, new protocols, and substantial reduction in maintenance costs to the U.S. military. The results from this study established the basis for detailed studies, thus knowledge gained will have broad implications in an array of industries.

Future Research: A key result of our one-year exploratory work, showed that further work is imperative to elucidate details of mechanical penetration and chemical degradation to support the mission, with the goal to provide effective remedial measures for US Army coatings degradation by mold fungi.

Keywords: Aircraft Coatings, Fungal Degradation, Corrosion, Polyurethane, Attenuated Total Reflectance- Fourier Transform Infrared (ATR-FTIR) spectroscopy, Mechanical Penetration.

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ACRONYMS AND ABBREVIATIONS

A. pullulans	*Aureobasidium pullulans*
AFM	Atomic Force Microscopy
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared Reflectance
ENVI	Environment for Visualization
ESEM	Environmental Scanning Electron Microscopy
ESTCP	Environmental Security Technology Certification Program
IDL	Interactive Data Language
IR	Infrared Spectroscopy
LSCM	Laser Scanning Confocal Microscopy
MNF	Minimum Noise Fraction
PCA	Principle Component Analysis
PDA	Potato Dextrose Agar
SERDP	Strategic Environmental Research & Development Program
SON	Statement of Need

Discussion of the Objectives and Criteria for the Success of the Exploratory Project

The overall goal of the project was to elucidate various mechanisms that mold fungi utilize to degrade various coating systems used on the surfaces of military aircraft/hardware. **The technical objective** was to investigate the mechanisms of mold fungi degradation to coatings on aircraft structures caused by mechanical and/or chemical penetration. These objectives were fulfilled by undertaking tasks, including A) testing of mold fungi on various coating materials that are used for coating systems (topcoat and primers); B) investigating direct penetration of mold fungi to topcoat and primers and associated physical changes leveraging atomic force microscopy (AFM) and laser scanning confocal microscopy, and C) utilizing infrared (IR) imaging system for the chemical composition of coating materials and its application for chemicals produced by mold fungi and during colonization to coating material along with effects of antifungal and antimicrobial peptides on mold fungi.

The SERDP Statement of Need (SON) was stated as: “understanding coating degradation mechanisms induced by fungi (mold/mildew)” with the objective of developing an understanding of the degradation mechanisms of coatings materials induced by fungi, primarily those induced by mold and mildew. During the project, the research team was successful in identifying and isolating a limited number of fungi, which had caused degradation on the US Army supplied coated samples.

The completion of these tasks generated proof of concept data and assisted with the design and development of fungal incubation system leading to coating degradation along with associated protocols. These, therefore, will form the basis for further detailed studies, thereby reducing risk through design and development of resins and additives that will enable environmentally friendly materials to perform as designed without the added problems associated with a microbial attack. This ability is paramount to the ultimate implementation of environmentally friendly finishing systems throughout the Department of Defense.

1 BACKGROUND

Degradation of aircraft coating systems is of critical importance from the aesthetic as well as safety concerns and causes a significant cost to the aircraft industry. The cost of metal corrosion in the United States is about $300 billion, of which $13 billion is incurred on aircrafts repairs alone (Simpson and Brooks, 1999). Current aircraft coating system can last for 6-8 years in services (Tiong and Clark, 2011). The layer that is first subjected to damage is the topcoat. The topcoat is very sensitive to temperature, moisture, ultraviolet (UV) radiation and mechanical damage such as cracking. The cracking is the primary sign of coating deterioration (Kim and Nairn, 2000). This may induce component failure through corrosion and/or cracking. For example, cracking of the coat may allow moisture to accumulate in the cracked groove and beneath the surface of the topcoat and allow fungi and bacteria to grow and penetrate both the topcoat and primer accompanied by connected degradation.

Bacteria and fungi have been associated and/or isolated from deteriorated inner surfaces of military aircraft (Gu *et al.* 2011). The role of fungi as an inducer of degradation of both metals and polymeric materials that have been used for surface coating of military aircraft have been documented (Tiong and Clark 2011).

The most common molds isolated from military aircraft are *Aspergillus, Penicillium, Fusarium, Cladosporium, Alternaria, and Mucor* (Little *et al.,* 1997a, and 1997b). For molds to grow and reproduce, they need only a food source – any organic material, such as aircraft coating materials, plant leaves, vegetable oil, diesel oil, wood, paper, or dirt or salt and - moisture (relative humidity > 60%). For example, for the polyurethane topcoats; previous reports indicated that mold fungi penetrated the polyurethane and formed a network of etched lines with no apparent degradation of polyurethane (Little *et al.* 1997b). Several environmental conditions prevail in the bilge areas of military aircraft operating in maritime environments enhance mold to grow and colonize those areas (Little and Ray, 2002; Lee *et al.* 2014). Such conditions exist due to the presence of moisture from water damage, excessive humidity, condensation, water infiltration, and the presence of salt or diesel oil. Under these conditions, *Cladosporium resinae* caused corrosion of 2024-T3 Aluminum (Al) Alloy (Little and Ray, 2002). The fungus exerted organic acid byproducts that selectively dissolved or chelated the copper, zinc, and iron at the grain boundaries of aircraft aluminum alloys, forming pits under fungal colonies. One of the most common US Air Force (USAF) aircraft alloys is Al 2024 T-3, which is known to be quite susceptible to corrosion (Bierwagen and Tallman, 2001).

Several mechanisms have been proposed for fungi induced corrosion in metals, their coating systems, and in non-metallic materials (Little and Ray (2002). These corrosion mechanisms include (i) local acid production e.g. citric and oxalic acids, (ii)

increase the acidity in their growing media, (iii) direct penetration and degradation of coatings, (iv) dissolution of protective greases, (v) under deposit corrosion, and (vi) metal concentration cells. Mold fungi also produced carbon dioxide, which reacts with water and forms carbonic acid. The production of acids penetrate the protective coating and cause it to tear. They also reported several fungi penetrated polyurethane used as a topcoat, but not the chromate primer (Little ,, 1997a, and 1997b). It is also evident by an earthy or musty smell in the suspected area of growth. It has been reported that mold fungi produced volatile organic compounds (VOC). VOC are products of the microbes' primary and secondary metabolism. In primary metabolism, the organism breaks down materials in the environment to extract nutrients needed for the maintenance of cell structures and, in the process, creates VOC as by-products. In secondary metabolism, the production of VOC is driven by the competition for resources in a nutrient-poor environment.

In this report, first various mold fungi species were isolated from corroded environmental samples, which were used for setting up fungal degradation experiments. The fungal isolates were identified based on the existing literature and fungal growth conditions were optimized. Then Raman and FTIR spectroscopy techniques were used for chemical characterization, isolated pure fungal species (and commercially available isolates), and various coating systems (in-house prepared as well as US-Army supplied). Finally, fungal degradation experiments were conducted using Raman and FTIR spectroscopy techniques for the assessment of chemical degradation of various coating systems and AFM, ESEM, and laser scanning microscopy techniques for the assessment of physical penetration/cracking of coating systems.

P.S. The sequential order of objectives and results presented in this final report do not match with those initially written in the proposal. All figures are provided in a separate file. With prior approval from the DOD SERDP Program Manager, for this study, we conducted experiments on readily available samples/coupons provided by the US Army.

2 MATERIALS AND METHODS

2.1 Isolation and Growth of Fungi

Several fungi species were isolated from locally available deteriorated paint samples on Potato Dextrose Agar (PDA) medium. For optimal growth, the inoculated PDA media in Petri-dishes were incubated at 28^{o}C. Typically, the fungal growth was observed in the Petri-dishes within 1 week of inoculation for all isolates as shown in **Figure 1A**. After visual observation of fungal growth, a bunch of hyphae were carefully taken out from the media and were laid out on a glass microscopic slide and covered with a coverslip. Then the coverslip was sealed from the sides to minimize the drying of samples as well as to prevent the spreading of the fungi. These slides were then observed under Zeiss Axio Imager M2 microscopy system with 20x (0.8 NA) objective in transmission mode for

optical characterization and identification of different isolates and images were captured for the identification of fungal species (**Figure 1B-D and Figure 3**).

In addition, two pure fungal isolates, *Aspergillus niger* and *Aureobasidium pullulans* were also obtained from commercially available microorganisms' culture bank-ATCC. These isolates were similarly grown on PDA agar media and optically characterized as described earlier.

2.2 Coating Systems

Two in-house prepared and three US Army provided coating systems were used in this study **(Figure 2).** The details of all five coating systems are given below. Additional information on the US Army coating systems is also provided in Table S1 in the supporting information.

- Coating system 1: Prepared In-house on a thin layer (~100 nm) Al-coated glass slides
  - Coating A- White primer (Rust Oleum®)
  - Coating B- While primer (Rust Oleum®) + clear topcoat (Dupli-Color®)
- Coating system 2: US Army supplied samples
  - Coating C- White primer
  - Coating D- While primer + Olive green topcoat (solvent based)
  - Coating E- While primer + Olive green topcoat (water based)

Hypothesis

Our hypothesis was that fungal infection causes damage to military hardware coatings. At this point, there are no studies validating this for mold fungi. Generally, the literature has primarily focused on bacterial damage as the cause of degradation. However, detailed studies on fungal damage are lacking, which makes this work significant.

2.3 Fungal Degradation of Coating Systems

Objective A: To test and visualize the influence of fungi on various coating systems (topcoat and primers). Please note that the objectives given herein are not sequential.

The testing and visualization of the fungi are achieved through inoculation of fungal propagules e.g. spore suspensions or plugs of fungal growth are applied to surfaces (e.g. coating primers, Whatman filter papers no. 2, potato dextrose agar (PDA) plates). Then the sample is incubated at optimal conditions to induce degradation and then visualized at discrete intervals. Our previous work has included the characterization of different

isolates of causal agent of wheat pathogens and DNA sequencing of fungi for plant transformation for mitigation of fungal damage (Fouly *et al.,* 2011 and 2012).

The coating systems degradation experiments were performed with various isolated and purchased fungal species grown on the PDA. First, various fungi isolates were inoculated on PDA media in separate Petri dishes and incubated at 28^0C for seven days. Multiple Petri-dishes were prepared from each isolate. After fungal growth in Petri-dishes small substrates (1 cm x 1 cm or larger) coated with different coating systems were treated with a surface disinfectant with 1% Clorox, washed 3 times in sterile water, plot dried on sterile dry tissues, and then placed in Petri-dishes containing pre-grown fungal isolates at three to four substrates per petri dish. Then these Petri-dishes were sealed and were similarly incubated at 28^0C and 80% relative humidity to enhance fungal growth as well as induce degradation process over 8-10 weeks. Control experiments were also run in parallel, where substrates with different coating systems were similarly processed and incubated without any fungal inoculation in separate Petri-dishes. A representative fungal degradation experimental set up is shown in **Figure 3. Accomplishment** constitutes the establishment of the infection process comprising of adhesion, pre- and post-penetration observed through the use of advanced instrumentation (e.g. ESEM).

2.4 Fourier Transform Infrared Spectroscopy (FTIR)

Cary 620 FTIR Microscope (Agilent Technologies) imaging system was used to collect FTIR data at 4 cm^{-1} spectral resolution. For pure fungal isolates characterization, a bunch of hyphae from the PDA media were laid onto low-E glass slides (Kevley Technologies) followed by 4% paraformaldehyde fixation. FTIR imaging data was collected in transflection mode between 3800 cm^{-1} to 900 cm^{-1} wavenumbers with 5.5 µm x 5.5 µm per pixel spatial resolution. Spectra for different strains were collected using identical spectral and spatial parameters with 128 spectral co-additions for background and 64 co-additions for the sample.

For degradation assessment on various coating systems, the FTIR data was collected with Attenuated Total Reflectance (ATR) attachment (Cary 630 FTIR Spectrometer). All the data collection parameters were similar to those described earlier, except that the ATR-FTIR was used with spatial resolution at 1.1 µm x 1.1 µm per pixel with point mode scan instead of the imaging mode scan. First, one substrate from each coating system was analyzed before the fungal degradation experiment. Three randomly selected areas of 70.4 µm x 70.4 µm each were chosen for the data collection per substrate. Data was similarly collected on substrates after the fungal degradation experiment with one substrate sample from each coating system-fungal isolate combination. Finally, to obtain more insight into the fungal degradation process spatial

distribution of the presence of coating materials with respect to hyphae, data was collected in ATR-FTIR image scan mode on selected substrates, such as *A. Pullulans* exposed coating system A.

Finally, all the FTIR data collected was preprocessed using minimum noise fraction (MNF) using Environment for Visualization (ENVI)- Interactive Data Language (IDL) version 4.8 (Reddy and Bhargava, 2010). The preprocessed data were then imported in MATLAB using customized algorithms for further data analysis.

2.5 Raman Spectroscopy (RS)

For Raman characterization, a confocal backscattering spontaneous Raman instrument (Horiba Scientific, LabRAM HR 3D) equipped with a Horiba Synapse back-illuminated deep-depletion CCD camera was used located at the Beckman Institute for Advanced Science and Technology. A 532 nm, 50 mW laser (CW Nd: YAG diode) was used for excitation and Raman spectra in the range of 300-3400 cm^{-1} wavenumbers. A long working distance 100 X objective (Leica, NA=0.8) was used with a 1 sec 5 accumulations signal integration time. For fungal pure culture characterization, fungi isolates grown on PDA agar medium were similarly prepared on a glass slide as mentioned earlier for optical characterization. The spectra were collected by scanning x-y grids on sections of hypha tips of various fungi at a resolution of 2 µm. To characterize fungal degradation using Raman spectroscopy, a strategy similar to the one described above for the FTIR was used. Raman data for substrates before and after exposure to fungi were collected at three random locations as described earlier using parameters used for fungal characterization. All spectra obtained were pre-processed using customized written code in Matlab 2016b (Mathworks®) in order to remove the background and for other preprocessing.

2.6 Environmental Scanning Electron Microscopy (ESEM)

FEI (Thermo Scientific) Quanta FEG 450 ESEM. ESEM work was conducted at the Beckman Institute for Advanced Science and Technology. The ESEM was used to determine the adhesion of fungi and subsequent etching of the samples. Fungal penetration into the coated surface was investigated. Initial observations showed that fungi can cause substantial damage to coated surfaces under conditions conducive to fungal growth.

2.7 Atomic Force Microscopy (AFM)

ASYLUM RESEARCH MFP-3D AFM feature closed-loop, low noise, high precision scanners, with Q-controlled AC modes (with phase imaging), piezo response imaging,

contact mode with lateral force, and detailed force-distance measurements were conducted at the Materials Research Laboratory. These systems allow scanning in the air or liquid environments and have extensive nanomanipulation and nanolithography capabilities. Maximum lateral scan size on these instruments is 90 µm x 90 µm with a maximum vertical range of 15 µm.

2.8 Laser Scanning Confocal Microscopy (LSCM)

Keyence VK-X250-3D optical profiler located at the Materials Research Laboratory was used. The Laser microscopes can perform non-contact profile, roughness, and film thickness measurements with nanometer-level resolution on any material or shape. Unlike interferometers or other 3D measurement systems, even objects with steep or curved surfaces or those that do not reflect light very well can be accurately measured. By combining white light with a laser light source laser scanning microscopes are able to scan a surface and collect both an optical image and high-resolution surface data. Nanometer-level heights can be measured by analyzing the intensity of the returned laser light relative to the z-position of the laser.

3 RESULTS AND DISCUSSION

3.1 Morphological and Chemical Characterization of Fungal Isolates

This section discusses the results of investigations into the mechanisms of mold fungi degradation to coatings on aircraft structures caused by mechanical and chemical penetration.

3.1.1 Optical Microscopy Characterization

Usually, there are two basic structural units of a typical fungi species, hyphae, and spores (Walter *et al.,* 2010). Hyphae, which are a large part of the entire fungal body, are usually slender filaments that form thick hyphal mat called mycelium, which is visible to the naked eye. The cylindrical hyphae are filled with cytoplasm with multi-nuclei and the hard cell wall is formed of Chitin (complex polysaccharides) that is difficult to penetrate except at the fungal tip. The spores, on the other hand, contain dehydrated cytoplasm and have hard protective coatings. The spores are responsible for spreading and germination of the fungi.

Filamentous fungi (e.g. mold fungi) grow radially in search of nutrients. The cells grow apically from the hyphal tips. Pressure-induced hyphal penetration of solid materials is generally accompanied by different chemical interactions. As the hyphal tip exerts itself into the environment it oozes substances, which soften or even dissolve material along its pathway. This combination of hyphal tip growth and chemical release is the very essence of the fungal organism; whereas, everything else is secondary to the process.

A slide culture is the best method for observing sporulation characteristics of most fungi for identification purposes, though it is still difficult to discern separate fungus.

To identify the different fungal species isolated from the environmental samples we examined the microscopic images taken from different isolates **(Figure 1B-D).** The morphological features of hyphae and spores from these images were compared with the existing literature (Brand, 2011; Barry and Williams, 2011). In this study, we isolated four fungal species viz. *Aspergillus niger, Fusarium spp, Alternaria,* and *Aeurobasidium pullulans*. The optical images obtained from the known cultures from FCCP are in agreement with the existing literature morphologies for *Aspergillus niger* and *Aureobasidium pullulans* (De Hoog and Yurlova, 2994; Domsch and Anderson, 1993).

3.1.2 Chemical Characterization of Fungi: Raman and FTIR Characterization

Average Raman spectrum of one of the fungal strain, *Fusarium spp.*, is shown in **Figure 4A** with a peak assigned to various biochemical components present in the hyphal tip in the fingerprint region of the spectra. The most prominent Raman peak centering at 1653 cm^{-1} due to amide-I bands results from the superposition of proteins, lipids, and polysaccharide vibration. It is hence the most prominent marker in the cell material; therefore, it is typically used as a reference band fungal cell matrix for predicting hyphal matrix distribution in *Schizophyllum commune (*Walter *et al.*, 2010). The Raman band with the peak centered at 1300 cm^{-1} is due to cytochrome ring mode that is coupled with the peripheral vinyl groups. Using these and other Raman peak assignments to various biochemical materials in cells coupled with the variations in peak intensities, a biochemical quantification of cell materials and its distribution for various fungi is possible.

Representative Raman and FTIR spectra for Coating A are shown in **Figures 5A and 5B**, respectively. The Raman spectrum for the in-house prepared coating A showed spectral signature similar to that reported for Auto paints in the literature (De Gelder, *et al.*, 2005). For example, Raman peaks centering at 446 cm^{-1} and 609 cm^{-1} represent the presence of rutile (TiO_2), which is a common component in the primers (De Gelder *et al.* 2005, Fidoac *et al.* 2006). The doublet near 1580 cm^{-1} and 1606 cm^{-1} in all three coating materials may be attributed to a quadrant stretch, which suggests the use of benzene derivatives in the production of the epoxy binder. Furthermore, the CH_2 twisting and rocking vibrations, CH_2 in-phase twisting vibrations, and the CH_3 and CH_2 deformations cause bands in the regions 1175–1310 cm^{-1}, 1295–1310 cm^{-1}, and 1446–1473 cm^{-1}. The presence of other Raman peaks in the spectrum showed presence of polyurethane, a common material used in all types of coating manufacturing (Parnell, Shane *et al.*, 2003). The Raman peaks at 1732 cm^{-1}, 1445 cm^{-1}, 1303 cm^{-1}, and 1185 cm^{-1} can be attributed to Ester υ(C=O) and urethane-I υ(C=O), υ_{sym} (N=C=O) and §(CH_2), §(CH) and urethane III, and urethane amide respectively.

An Attenuated Total Reflectance with Fourier Transform Infrared (ATR-FTIR) spectra of coating A in **Figure 5B** showed several sharp bands. Many of these bands are characteristic of typical military coating systems previously reported by Keene *et al.*, (2004) and Nguyen *et al.* (2002). Some of the interesting bands reported were at 1726 cm^{-1} for C=O stretch in ester, 1240 cm-1 for (C=O) + (O-CH2) stretch in Ester and 1147 cm^{-1} for C-O-C ester stretch. The interesting ester bond around 1730 cm^{-1} in both Raman and FTIR spectra will be used for the characterization of coatings degradation in this system.

3.2 Fungal Degradation Assessment of Coating Systems

This section covers chemical and mechanical degradation studies involving in-house prepared and US Army provided coating samples using two distinct spectroscopic techniques viz. Raman and ATR-FTIR.

3.2.1 Chemical Degradation

Objective C: To develop infrared (IR) imaging system for the chemical composition of coating materials and its application for chemicals produced by mold fungi and during colonization of coating material along with the influence of antifungal and antimicrobial peptides on mold fungi.

This section covers chemical degradation studies using two distinct spectroscopic techniques viz. Raman and ATR-FTIR.

3.2.1a. Raman Spectroscopy: Microscopic observation of the fungal exposed substrates showed growth of fungal mat on the top of the coatings. However, as expected no such growth was observed on the control samples as shown in **Figure 6** for *Fusarium spp.* grown on coating A after 10 weeks of exposure. For quantitative assessment-, Raman spectra were collected for 3-areas randomly selected on the control and fungal exposed samples with the average spectra shown in **Figure 7 A**. Though the differences in the spectral features were minimal for the exposed and control samples, the principal components analysis (PCA) showed a clear separation between the two samples. Further analysis of PC1 loadings (data not shown) showed the separation was based on Raman peak intensities at 1732 cm^{-1} and 1445 cm^{-1}, concurrently with large contributions from silent regions of the spectra. In addition, as was observed from **Figure 7B** that the separation in the data is only along the PC1 axis, which accounted for only 52% variance. Based on these results it was difficult to point out the specific mechanism of the fungal degradation, considering longer scanning time from small areas for spontaneous Raman analysis used in this study; therefore, FTIR evaluation of the fungal degradation was considered in the next step.

3.2.1b. ATR-FTIR Spectroscopy: The representative results obtained for coating A and coating B systems exposed to *Aeurobasidium pullulans* (*A. pullulans*) are shown in **Figure 8.** Chemical relevance of various bands present in coating A (and similar were in coating B) is shown in **Figure 5B** as discussed earlier. The most critical for the coating degradation assessment were the peak at 1726 cm-1 corresponding to C=O bond in ester and Urethane Amine I bond (Parnell *et al.*, 2003) and Amide I band between 1635-1650 cm-1 observed for chitin obtained from the fungal origin (Wysokowski *et al.,* 2015). **Figure 8A** showed average FTIR spectra for coating A and coating B control and *A. Pullulans* exposed samples. In both sets of spectra, the intensity of ester C=O band at 1726 cm^{-1} decreased with the increase in the Amide-I band at 1635-1650 cm^{-1} in fungi exposed samples as compared to the corresponding control spectra. These results indicated that the exposure to *A. Pullulans* for 10 weeks had resulted in the degradation of both the coating systems. Results also showed a large reduction in 1726 cm^{-1} peak intensity in coating A (i.e. primer only coated samples) as compared to coating B (i.e. primer+tc coated samples). In addition to 1726 cm^{-1} Carbonyl (C=O) species, other bands relevant to polyurethane also showed a decrease in intensity, such as 1466 cm^{-1} for Aliphatic (CH_2) stretch polyurethane backbone; 1240 cm^{-1} for (C=O) + (O-CH_2) stretch, and 1147 cm^{-1} for C-O-C stretch. The PCA analysis of the FTIR data from control and fungal exposed samples also showed very clear separation with PC1 accounting for 98 % variance in the samples (Figure 8B). This prima facie suggested a faster degradation rate with primer only coating; therefore, the topcoat provided protection from fungal degradation. However, this may also be a function of fungi density on the sample, which is indicated by a relatively higher 1635-1650 cm^{-1} intensity in the coating A. Thus, a further careful investigation is required to achieve a concrete conclusion. The results from the fungal degradation experiment with two other fungi isolates, *Aspergillus niger* and *Alternaria* on coating B showed a minimal reduction in ester band at 1726 cm^{-1} indicating less noticeable degradation.

Thereafter, the spatial distribution of the *Fusarium spp.* hyphae on primer only coating and its influence on the distribution of the primer itself were characterized by plotting FTIR band intensities at 1645 cm^{-1} and 1726 cm^{-1} respectively, comparing it with the bright-field microscopy image of the same area **(Figure 9)**. It was observed that the FTIR fungal distribution (Figure 9B) matched well with the actual bright-field microscopy image (Figure 9A). In addition, the distributions of fungi and primer coatings were complementary to each other, i.e. the presence of fungi is marked by the low concentration of the primer and vice-versa. These observations indicated that the presence of fungi caused degradation of coating material, thus depleting primer concentration in the area(s) where fungi grew.

Fungal degradation experiments and FTIR degradation assessment were also carried out for the US Army provided substrates, i.e. coatings C, D, and E. Representative results obtained for coating D are shown in **Figure 10.** The reduction in the FTIR bands for fungal exposed samples compared to control samples is evident for siloxane in the fingerprint region (Keene *et al.* 2004) and as well as peaks in CH regions. The PCA analysis further showed clear separation in fungal exposed and control samples with PC1 accounting for 78% variance. Thus, these results showed the potential of FTIR in assessing chemical degradation in the military aircraft coating system.

Chemical Imaging Accomplishments:

Raman spectroscopy and IR spectroscopy were used for investigation and characterization of the following aspects:

Raman spectroscopy:

1- Several mold fungi from environmental degraded paint samples and pure isolates from ATTC.
2- Chemical components present in different in-house prepared coating systems.
3- Fungal degradation of different in-house prepared coating systems.

Infrared spectroscopy:

4- Mold fungi from environmental degraded paint as well as ATCC pure isolates from ATTC.
5- Chemical components present in different in-house prepared coating systems.
6- Chemical components present in US Army supplied coating systems.
7- Fungal degradation of different in-house prepared coating systems.
8- Fungal degradation of US Army supplied coating systems.
9- Applied chemometric approaches, PCA and PLS analyses, for fungal degradation assessment.

These accomplishments met the objective of developing of an infrared (IR) imaging system for the chemical composition of coating materials and its application for chemicals produced by mold fungi and during colonization of coating material along with the influence of antifungal and antimicrobial peptides on mold fungi. These accomplishments have provided the foundational basis for further in-depth studies.

3.2.2 Mechanical Penetration and Damage to Coating Surfaces

Objective B. To investigate direct penetration of **mold fungi** to topcoat and primers and associated physical changes using atomic force microscopy (AFM).

We hypothesized that the first occurrence of coating system degradation by mold fungi is the direct/mechanical penetration of the topcoat. This process induces localized pores that may lead to accumulation of water and these pores

consequently aid the fungi to produce chemical changes (hydrolyzed enzymes, acids, etc.) that further enhance the degradation of primers and cause corrosion to metal coupons. To prove our hypothesis, we used atomic force microscopy (AFM) to study whether the mold fungi and *Aureobasidium pullulans* used force to penetrate topcoat and primers. Until now, very few studies have addressed the mechanical attack or direct penetration of mold fungi to both epoxy primers and/or epoxy and polyurethane topcoat.

3.3.2.1. Reflected Light Microscopy (RLM): RLM was used to examine the fungal growth on painted materials (Coat systems 1A and 1B; see materials and methods section) that were usually inaccessible to conventional transmitted light techniques. Painted surfaces are considered opaque if a thin (polished or not) section about 25 micrometers in thickness is non-transparent in the visible light spectrum range between 450 and 650 nanometers. Presented in **Figure 11** are reflected light digital image 20X revealing details of coating system 1A without fungi as a control. **Figure 12** presents reflected light digital images revealing details of hyphal growth of both *Alternaria* and *Fusarium* on the surface of A) coating system 1A and B) coating system 1B. Rusty colored appeared on both A and B. This could be attributed to either the spores from both of the fungi along with some chemicals produced by the fungus or because of fungal paint surface chemical interaction. The metrics for accomplishment included success in inducing the damage to coating surfaces at laboratory conditions.

3.3.2.2. The Use of Atomic Force Microscopy (AFM): Different fungal isolates were used to inoculate coating systems 1A and 1B (see materials and methods section) and incubated at 28ºC and at 80% relative humidity for 20 weeks. Control (un-inoculated) and inoculated samples were scanned using atomic force microscopy (AFM). **Figures 13 and 14** showed scans of control coating system 1A (auto-primer on an Aluminum coated slide). Some pits and an elevated area along the surface of the paint were observed. **Figure 15** (A and B) showed an AFM scan of inoculated coating systems 1A and 1B with both *Alternaria* (Fig. 15.A) and *Fusarium* (Fig. 15.B). These images reveal the presence of valleys and hills on painted surfaces. These hills were observed near the edges of the inoculated slides where the initial inoculum was added with fungal growth expanding toward the middle of the painted surface. The height varied from -10nm (small pits) to 250nm superficial hyphae of the growing fungi on coated surfaces. Fungal growth and infection of coating surfaces (system 1A and 1B) increased surface roughness and impeded the AFM tips from scanning these surfaces. Future research may include AFM scan at earlier stages of infection (few hours to few days post-inoculation by both *Alternaria* and *Fusarium*).

3.3.2.3. The Use of Laser Confocal Scanning Microscopy (LCSM): **Figure 16** represents an image captured by a laser scanning confocal microscope of auto coat system 1A (Al-coated glass slides with protective layers of a white Universal bonding primer (Rust Oleum®). It was infected with *Aeurobasidium pullulans* after removing the fungus with soft lens tissues (using hand friction). The figure showed

the topological spread with two distinct areas, one infected with the fungus (left with dark color) and the other area (right with white color) not infected with the fungus. It showed the fungus was able to penetrate the surfaces and lift the coat along with a consortium of chemical materials above the surface during penetration and degradation (thereby causing changes in the topology of the coated surface).

3.3.2.4. The use of Environmental Scanning Electron Microscopy (ESEM): Toward the end of 4th Quarter, we had received cleaned samples from the US Army (the coating system 2 described in materials and methods section). There was enough evidence from the aforementioned description that *Alternaria alternata, Fusarium* sp. and *A. pullulans* infected the coating system 1 and caused mechanical penetration and damage to coating 1A and 1B. In the following section, spore suspension of A. pullulans and consortium of isolated fungi from the environment were used, as described in the Materials and Methods section. The purpose of it was to reduce the incubation time to induce mechanical penetration and damage to coating system 2. A description detailing the methods used to produce spore suspension, inoculation, and incubation time is provided below. It is followed by a description on the use of ESEM to scan the interaction of germinating spore and fungal hyphae with coated surfaces.

3.3.2.4a *Inoculation of coating surfaces with different fungi and speed up the deterioration of coating surfaces:*

Spore production: Subculturing mycelial plugs onto Petri dishes of potato dextrose agar (PDA) induced reproductive growth of *Fusarium, Alternaria, Aspergillus, Epicoccum,* and *Penicillium*. Petri-dishes were incubated at room temperature in the dark until sporulated. Sporulation was determined by cutting a small portion (approximately 1 cm × 1 cm in size) of a mycelial block of the cultures. Using a sterile 100-µL pipette tip, 75 µL-distilled water was applied on the surface of the mycelial block by sucking up and down using the pipette several times. Then, a drop of the washing solution was observed under a light microscope to detect the presence of conidia.

Preparing spores for inoculation of coating surfaces: Sterile distilled water was added aseptically to the PDA plates, which were then placed on an orbital shaker operated at 38-× g for 20 mins to liberate spores from the mycelia. The spores were counted using a hemocytometer and an appropriate dilution was made to obtain a final concentration of 100 spores/mL. Viability was confirmed by spreading 300 µL of a diluted spore suspension onto a potato dextrose agar plate. The plate was incubated at room temperature in the dark for 24 h to germinate the spores, and then the number of colonies were counted and compared with the total spores plated.

Inoculation and incubation: Coating surfaces were sterilized using 1% sodium hypochlorite (Chlorox) for 2-3 minutes, then washed three times in sterilized distilled water and blot dry on sterilized tissues. Appropriate spore density was applied to coating surfaces, spore concentrations (1 × 103, and 1 × 104 spores/mL). Coatings were placed inside culture plates, which were incubated at 28 C for 3 weeks to induce discoloration of coating surfaces.

3.3.2.4b Scanning electron microscopy (SEM): Environmental Scanning Electron microscopy (ESEM) (FEI Quanta) was used to determine (a) the growth of fungal strains on coating system 2 and (b) the degradation/damage caused by fungi on the coating surfaces. At the end of 3 weeks incubation, the coating system was analyzed. For SEM analysis, samples were fixed with 4% paraformaldehyde, washed 3 times with sterile distilled water, air-dried and sputter coated with gold-palladium alloy. Results of the experiments carried out with inoculated US Army coating system (primer only, coat 1 and coat 2; see Materials and Methods for complete description of the system) in laboratory conditions are shown in Figs. 17, 18, and 19. After 3 weeks of incubation, the degradation phenomenon for the coating system 1 caused by the growth of fungi were clearly visible (Figs. 17, 18, and 19). Fungal growth was observed along the surfaces of primer, coats 1 and 2. Due to the fungal sporulation and colonization, coating surfaces changed in appearance and were fragmented by the hyphae penetration. It was also observed that in some cases the surface was dry and cracked (Fig. 17, and 18 respectively). ESEM scan of US army primer and coat 1- inoculated with *Aeurobasidium pullulans* is shown in **Figure 20**. Spores are produced from the fungal mycelium growing on the surface. The figure shows *A. pullulans* white mycelium colonize coat 1 surface, change surface appearance and caused cracking.

Mechanical Penetration and Damage to Coating Surfaces Accomplishments:

1- Isolated several mold fungi from environmental degraded paint samples.
2- Isolated several mold fungi from cleaned US Army coating systems (unusual).
3- Characterized mold isolates isolated from environmental degraded paint samples.
4- Partial identification of mold isolates from cleaned US Army coating systems.
5- Tested the ability of isolated fungi to grow and colonize surfaces of in-house prepared model coating systems.
6- Optimized conditions for colonization of coating surfaces by mold fungi.
7- Demonstrated the ability of *Alternaria, Fusarium* and *Aeurobasidium pullulans* fungi to penetrate, grow and cause physical damage to in-house prepared model coating systems coating surfaces (using Reflected light microscopy, AFM and confocal scanning laser microscope).
8- Partial demonstration of each of *A. pullulans* and consortium of mold isolates on US Army coating systems (using Environmental Scanning Electron Microscopy).
9- Demonstrated the ability to grow and colonize mold fungi and induce cracks to the US Army coating system.
10- Developed the capability to accelerate and analyze the degradation process involving mechanical penetration and chemical degradation.

We successfully demonstrated that the first occurrence of coating system degradation by mold fungi was the direct/mechanical penetration of the topcoat.

4 CONCLUSIONS AND FUTURE RESEARCH

In the current study, microscopic images of different isolates were examined using an array of imaging tools to identify the different fungal species isolated from the environmental samples. Four fungal species were isolated viz. *Aspergillus niger, Fusarium spp, Alternaria,* and *Aeurobasidium pullulans*. The optical images obtained from the known cultures from FCCP were in conformity with the existing literature morphologies for *Aspergillus niger* and *Aureobasidium pullulans*. However, knowledge gaps remain, since only a very limited number of fungi were tested. Therefore, in future studies, we will build on the experimental techniques developed in this project to isolate an array of fungal species for in-depth exploration of a broad range of fungi on military hardware.

Reflected Light Microscopy was used to examine the fungal growth on painted materials (coating systems 1A and 1B) that are usually opaque to conventional transmitted light techniques. Rusty colored appeared on both A and B. This was attributed either to the spores from the two fungi along with some chemicals produced by the fungus or due to the fungal coated surface chemical interaction.

Atomic Force Microscopy (AFM) was used on different fungal isolates to inoculate coating systems 1A and 1B. The images obtained showed valleys and hills on the coated surface. It was noticed that the hills arose near the edges of inoculated slides as the initial inoculum was deposited, with it the fungus stretched toward the center of the coated surface. The fungal growth and infection of coating surfaces increased surface roughness, impeding the scanning of surfaces using AFM tips.

Laser Confocal Scanning Microscopy (LCSM) was used to obtain images of auto coat system 1A (Al-coated glass slides with protective layers of a white Universal bonding primer (Rust Oleum®)) infected with *Aeurobasidium pullulans*. The images showed the topological spread with two distinct areas i.e. fungus infected and non-infected. Thus revealing surface penetration by the fungus showing a lift in the coated surface along with a consortium of chemical materials above the surface during penetration and degradation, causing changes in the surface topology of the coated surface.

Further work is necessitated to elucidate details of mechanical penetration and chemical degradation to support the mission, with the goal to provide effective remedial measures for US Army coatings degradation by mold fungi, leading to substantial savings in maintenance costs.

Exploratory research showed some evidence of contamination of the US Army supplied clean samples. This was surprising and intriguing to the research team, raising several questions. To help answer these questions, we would like to explore in depth:

- Why and how did the clean US Army samples inherit mold fungi?
- Was it inherent to the paint supplied by the paint company, or were it the spraying or drying techniques, or
- Were there shipping and handling issues, or were there any other factor(s)?

To answer these questions and others, we will investigate in depth the methods being currently used to prepare the paints and coatings, spray methods and ambient conditions of hardware used. As a side note during the 2017 Annual Meeting of SERDP/ ESTCP in Washington D.C., a presentation by a speaker from the US Army mentioned that they prepare paints and coat at acidic (PH) conditions, which were known to be favorable for fungal growth. DNA techniques will also be employed in the future to identify the isolated fungi from the US Army and other military hardware.

4.1 Future Research for Mechanical penetration and how to impede the process

To study the mechanical penetration process we used Environmental Scanning Electron Microscopy (ESEM) on clean samples from the US Army (coating system 2). There was evidence that *Alternaria alternata, Fusarium* sp., and *A. pullulans* infected the coating system, caused mechanical penetration, and damage to the coatings. A spore suspension of *A. pullulans* and consortium of isolated fungi from the environment were also used. The aim was to reduce the incubation time to induce mechanical penetration and damage to coating system 2. Fungal growth was observed along the surfaces of primer, coats 1 and 2. Due to the fungal sporulation and colonization, coating surfaces changed in appearance and were fragmented by the hyphae penetration. The ability to grow and colonize mold fungi and induce cracks to the US Army coating system was clearly demonstrated. In addition, the capability was developed to accelerate and analyze the degradation process involving mechanical penetration and chemical degradation.

Therefore, further detailed studies are needed to investigate the mechanisms of mechanical penetration to address the following questions:

- What causes a crack in topcoat during penetration (quantify the forces exerted by the fungi)?
- How does the fungus get underneath the coating? Is it hyphal tip penetration or a network of fungal mycelium?

We have the expertise to design the appropriate physical surfaces (thickness, roughness, dry/wet, different acidity levels) of coatings that slow down or impede fungal molds from causing the invasion of coating surfaces. We will study the attachments of different mold fungi (spores, germlings, and hyphal turgor pressure exerted) on different coated surfaces.

To answer these questions, time series involving ESEM, AFM, transmission electron microscopy, and force traction microscopic studies at various stages of the fungal growth attachment and penetration to coating surfaces will be used.

The role of the hyphal tip is critical to the degradation process; therefore, it requires further detailed investigation on the following:

- How are hyphal tips guided and controlled?
- What are the local mechanisms through which *A. pullulans* degrade the coating?
- Which fungi actually contribute to direct penetration of coatings?
- Do the morphological properties of hyphae regulated differentially depend on the coating composition and topology?

To understand clearly how hyphae behave in the host environment, we envisage combining genetic approaches with mechanobiology. These questions when answered will introduce new scientific nomenclature and in-depth understanding of fungi-coating surface interface.

What type of investigation is needed to reduce chemical damage to coatings? To eliminate or minimize the impact of mold fungi on coatings, infrared imaging of interaction of mold fungi with various antifungal, yeast, and antimicrobial peptides integrated into topcoats or primers used to coat aluminum alloys used in military aircraft/hardware were used.

4.2 Future Research for Chemical degradation by mold fungi:

To study chemical degradation two distinct spectroscopic techniques i.e. Raman and ATR-FTIR were used.

Attenuated Total Reflectance with Fourier Transform Infrared (ATR-FTIR) spectra of coatings showed several sharp bands characteristic of typical military coating systems. The noteworthy ester bond around 1730 cm^{-1} in both Raman and FTIR spectra enabled coatings degradation characterization for the system.

ATR-FTIR Spectroscopy yielded representative results for the coating systems A and B, which were exposed to *Aeurobasidium pullulans*. The results showed that the exposure to *A. Pullulans* for 10 weeks had resulted in the degradation of both coating systems. The PCA analysis of the FTIR data from control and fungal exposed samples also showed very clear separation with PC1 accounting for 98 % variance in the samples. This prima facie suggested a faster degradation rate with primer only coating thus topcoat provided protection from fungal degradation. However, this may also be a function of fungi density on the sample, which is revealed by relatively higher 1635-1650 cm^{-1} intensity in the coating A. Thus, a further careful investigation is required to arrive at a concrete conclusion.

The spatial distribution of the *Fusarium spp.* hyphae on primer only coating and its influence on the distribution of the primer itself were characterized by plotting FTIR band intensities at 1645 cm^{-1} and 1726 cm^{-1}. These observations indicated that the presence of fungi caused degradation of coating material, therefore depleting primer concentration in fungi growth areas.

Fungal degradation experiments and FTIR degradation assessment were also carried out on the US Army provided substrates (coatings C, D, and E). The PCA analysis showed clear separation in fungal exposed and control samples with 78% variance. Thus, these results showed the viability of FTIR in assessing chemical degradation in the military aircraft coating system. This will enable us to address the following questions in future studies:

- What are the specific components of the US Army coating systems?
- How is the surface degraded and which byproducts are formed during the process?
- What is the rate of degradation?
- How often the coats need to be removed and replaced?

To answer these questions we need to study thin coatings of US Army supplied and in-house developed coatings (thick coatings tend to have excessive scattering for IR as well as Raman). Identifying the kinetics of degradation (various time points) will be the key to our findings.

These efforts will be useful in designing remedial measures- such as alcohol or organic acid formation that will lower the pH. Will there be other implications for the coating system and the military hardware/aircraft material (Aluminum) itself.

Mold Fungi on military coating systems

The following procedures will be utilized to control mold fungi:

- Isolation and characterization of antagonistic microorganisms (i.e. bacteria, yeast, and nonpathogenic fungi) from US army coating systems, and the use genomic technology to characterize microbiomes on different coating systems

- Test antagonistic microorganisms for inhibition of mold fungi (*in vitro* and *in vivo*) and study the mechanism of inhibition.

- Develop safe biological agents for inhibition and to slow down the penetration of mold fungi to coated surfaces.

- Develop a portable, cost-effective IR instrument for on-site degradation process assessment (on the aircraft/military hardware), and can take remedial measures if the signs of degradation appear.

- The research team has expertise in building several in-house Raman and IR instruments (Bhargava, *et al.,* 2005, 2006, 2007, 2012), as well as fungal pathology (Fouly *et al.,* 2011, 2012), and in imaging, biosensing, mechanobiology, and systems integration (Ahmad, 1996, 1999; Rajagopolan and Saif, 2011).

In conclusion, the results of our one-year exploratory work laid the foundations for further work. Therefore, it is imperative to elucidate details of mechanical penetration and chemical degradation to support the mission; with the goal to provide effective remedial measures for US Army coatings degradation by mold fungi.

5 SPECIFIC OUTCOMES

Conference/Invited Presentations/Talks/Posters

- Singh, R., Hanafy M. Fouly, Rohit Bhargava, Irfan S. Ahmad**.** 2018. Characterization of fungal degradation mechanisms of organic coatings (polyurethane) using Fourier-transform infrared spectroscopy (FTIR). 256th ACS National Meeting, Boston, MA. August 19-23, 2018.

- Ahmad, I.S., H.M. Fouly, Rohit Bhargava, Taher Saif, Rajveer Singh. 2018. Mechanisms of Fungal Degradation of Military Aircraft Coatings. SERDP & ESTCP Winter In-Progress Review (IPR) Meeting and Symposium. Weapons Systems and Platforms. Feb. 27-28, 2018, Arlington, VA **(invited).**

- Fouly, Hanafy M.; Rajveer Singh; Rohit Bhargava; Taher Saif; Irfan S. Ahmad. 2017. Preliminary Study of Mold Damage on Coatings. Poster. Department of Defense Strategic Environmental Research and Development Program (SERDP) and Environmental Security Technology Certification Program (ESTCP) Symposium 2017, November 28-30, 2017, Washington, D.C.

- Singh, Rajveer; Hanafy M. Fouly; Rohit Bhargava; Taher Saif; Irfan S. Ahmad. 2017. Assessment of Fungal Degradation of Metal Coatings using Raman Spectroscopy. Poster. Sixth Annual MRL Fall Conference 2017- Biological and Materials, University of Illinois, Nov. 8-9, 2017, Urbana-Champaign, Illinois.

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6.1.1.1 Supporting Information:

Table S1: Details of US Army provided coating systems.

S.#	Coating systems	Coatings details (US Army): 2024 T3 Aluminum pretreated with Alodine 1200S
(i)	Coating C	Primer used on all panels: Sherwin Williams MIL-DTL-53022 TIV E90S228 6016-62356 exp: 11/11/17, batch: LM3166JJ; catalyst - V93V00235 650350705 exp: 5/17/18, Batch: XM1976ME
(ii)	Coating D	Waterborne Topcoat: Sherwin Williams MIL-DTL-64159 TII Green 383 F93G504 5012-34488, exp: 1/13/18, batch: LM0137EA; catalyst - V93V502 6016-18069 exp: 1/27/18, batch: MQ0277NC
(iii)	Coating E	Solvent Topcoat: Sherwin Williams MIL-DTL-53039 TIV Green 383 F93G0S116 6504-55470 exp: 11/03/17, batch: MQ3086KH

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