PLANT PATHOGENICITY IN SPACEFLIGHT ENVIRONMENTS

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ABSTRACT

Plants grown in microgravity are subject to many environmental stresses, which may promote microbial growth and result in pathogenicity to the plant. Recent plant experiments with super dwarf wheat aboard the NASA Space Shuttle and NASA/Russian Mir Space Station returned from the mission with severe degrees of fungal contamination. Understanding the cause of such microbial contamination and methods to eliminate it are necessary prerequisites for continued plant growth and development studies in microgravity. A seed-borne fungal endophyte, an *Acremonium* species was identified in the seed used in both the spaceflight missions and was also recovered from the leaf sheaths of the symptomatic super dwarf wheat that was flown on the 8-day mission aboard U.S. Space Shuttle Discovery (CHROMEX-06, STS-63). The super dwarf wheat infected with the *Acremonium* endophyte was symptomless when grown under greenhouse conditions. Our data suggest that growth in spaceflight conditions (high relative humidity, high atmospheric carbon dioxide, low light and available nutrients) can induce the transition in the fungus from a symptomless to a pathogenic state on its plant host. Plant growth under spaceflight conditions enabled this *Acremonium* species to escape its asymptomatic endophytic existence and become pathogenic on super dwarf wheat.

INTRODUCTION

Plant-microbe interactions have rarely been considered to be significant components of plant growth experiments thus far conducted in space. Clearly, the spaceflight environment is not sterile and microbes have been isolated from spaceflight hardware that are capable of colonizing plants and their environments (4,15,22,23). The few investigations, which have been reported in the literature tend to substantiate a general pattern of increased growth rates for microbial organisms exposed to altered conditions of gravity (7, 9,16,25).

The artificial conditions under which plants have been and will continue to be cultured in space may provide a greater opportunity for potential pathogens to colonize plants and reduce quality and yield. Plants grown under the controlled environments used in spaceflight experiments are subjected to environmental stresses, which include elevated carbon dioxide (CO_2), low photosynthetic photon flux density and high relative humidity. Several studies also indicate altered plant growth and metabolism in microgravity (6,8,24), which may result in increased plant stress and thus perpetuate more aggressive microbial growth resulting in pathogenicity to the plant.

This study originated as a result of the CHROMEX-06 spaceflight experiment, which was designed to investigate the effect of microgravity on the growth and development in wheat, cv. super dwarf (*Triticum aestivum* L). Despite an initial seed sterilization protocol, approximately 50% of the seedlings returned from the mission with visual signs of fungal contamination. We report herein on the disease status of the plants and studies conducted to understand the source of contamination.

MATERIALS AND METHODS

Plant Growth in Spaceflight Environments

Seventy-two super dwarf wheat plants were grown in a plant growth unit (PGU) in the middeck locker of Space Shuttle Discovery for an 8-day mission. The PGU housed six individual enclosed plant growth chambers (PGCs). Prior to planting, super dwarf wheat seeds were subjected to rigorous surface sterilization protocols (Table 2) and was then transferred to 4°C for 4 days to synchronize germination. Twelve 36-h-old germinated seedlings were planted (24 h prior to lift-off)

into individual nitex planting pockets which fit snugly into the foam base in each PGC. Foam root barriers were placed into each planting pocket on either side of the seedling.

Environmental parameters used for plant growth are described by Krikorian and Levine, 1992 (10) and Levine and Krikorian (1992) (11). Each foam base was supplied with 200 ml of sterile one-half strength Schenk and Hildebrandt nutrient medium (18) supplemented with 0.5% surcose prior to planting. Plants received a 24 h photoperiod with light intensities ranging from approximately 50-60 μmol m^{-2} s^{-1}. Relative humidity was approximately 90 to 100%. Carbon dioxide inside PGCs increased to 150,000 ppm by the end of the mission. Ground control plants received to the same environmental conditions as the flight plants (except for microgravity) supplied by an orbital environmental simulation chamber (OES) at Kennedy Space Center.

Plant sections consisting of leaf sheath and meristematic tip regions were cultured on potato dextrose agar (PDA, Difco) at the end of the mission to identify the microbial contaminants.

Isolation of Seed-Borne Microbes

The spring wheat, super dwarf (*Triticum aestivum* L) was initially obtained from CIMMYT Breeding Station, Mexico. Additional accessions were provided by Dr. Bruce Bugbee, Plant, Soils and Biometeorology Dept., Utah State University. Seed lot 2 was used in both the U.S. Space Shuttle (STS-63) and NASA/Russian Mir wheat experiments in 1995 and endophyte-free seed was generated by Bishop and Anderson at USU and designated as lot 5 (Table 1).

Table 1. Super dwarf seed lots examined for endophytic microbial contamination.

Seed lot	Year	Seed Use
1	1991	Preliminary studies
2	1993	Spaceflight experiments 1995
3	1993	Seed increased from lot2 (greenhouse)
4	1995	Seed distributed to NASA Centers
5	1995	Endophyte-free seed

Additional Surface Sterilization Treatments

Seed was immersed in concentrated sulfuric acid for 5 min or 30% hydrogen peroxide for 10 min, followed by extensive washing in sterile distilled water. The seeds were transferred to PDA plates, placed at 4°C for 4 days and then placed at 22°C under incandescent lighting to assess percent sterility and germination over an 8-day period (Table 3).

Fungal Identification

Seed-borne endophytes were recovered from super dwarf seed after rigorous surface sterilization (17, 20). Seeds were subsequently cultured on PDA, solidified with 0.8% agar at 22°C for a period of 2 weeks. The type of contamination, % sterility and % germination were recorded.

The endophytic fungi growing from the seed were isolated by transfer onto fresh PDA plates, grown for 2 weeks at 22°C and purified by single spore isolation. Conidial preparations were re-suspended in 5 ml of sterile double deionized water, filtered through silanized glass wool to remove any mycelia prior to inoculations. The fungal isolate was stored at -80°C in 15% glycerol either as a plug of mycelium from a PDA plate or as a pure spore suspension. Characteristics of the spores and mycelium were noted and used in the identification of the endophytic fungi using taxonomic references (13,26, 27).

Establishment of Disease Symptoms

Endophyte-free seed (Table 2) was inoculated with a suspension of *Acremonium* spores (10^6spores/ml) in 1% carboxy-methylcellulose for 5 min. The spores were obtained as described above from the 2-week *Acremonium* cultures on PDA.

Disease symptoms caused by *Acremonium* were examined and compared to those symptoms seen on plants from the CHROMEX-06 wheat experiment. Five seeds were transferred to each sterile Magenta box containing 200 ml vermiculite moistened with 100 ml of sterile water. The closed boxes were placed at 26°C under a 24 h photoperiod provided by incandescent lights for 8 days. As a control, the seed was treated with sterile water instead of the spore suspension. Three replicates were used for each treatment.

The potential for fungal dispersion from *Acremonium*-infected seed was evaluated by planting a single lot 2 surface-sterilized (yet endophyte-infested seed) into a nitex pocket in each of the six PGC containing 11 other endophyte-free seeds. The plants were grown under conditions similar to those used in spaceflight: high relative humidity; 85-90%, a stable temperature of 28°C and low incandescent lighting for a 24 h photoperiod. Observations on microbial spread and infection of neighboring plants were recorded daily (Figs. 1 and 2).

Microscopic Examination of *Acremonium* in Asymptomatic and Pathogenic Growth Habits

Characteristic growth of *Acremonium* mycelia and conidiospores on PDA was examined and compared to growth in both the asymptomatic and pathogenic growth habits of the fungus in association with the plant (Fig 4). Infection or the presence of the endophytic fungi and location in plant tissues was visualized microscopically (sections of leaves, sheaths and roots under 40X magnification) with an aniline-blue, lactic acid stain (1% aqueous) (2). Photographs were obtained using a 35 mm camera attachment with an Olympus microscope (Figs. 3, 4).

Composition of Cell Wall-Associated Peroxidases

Asceptic imbibed seeds were inoculated with pure spore suspensions of *Acremonium* species and *Fusarium culmorum* . They were grown for 8 days in sterile Magenta boxes as described above. Differences in cell wall-associated peroxidases from leaves, sheaths and roots from *Acremonium*-inoculated and non-inoculated plants were examined. *Fusarium culmorum*, a well characterized pathogenic endophyte on wheat was used as a positive control to examine changes in peroxidase activity and composition. Changes in total peroxidase activity were evaluated using methods by Albert and Anderson (1987) (1) and peroxidase composition was determined using isoelectric focusing (12). Because of the specificity of peroxidases to different substrates, gels were stained separately for peroxidase activity with 0.1% (w/v) hydrogen peroxide in Tris buffer (pH 7.5) using both catechol/p-phenyldiamine and chloronapthol-based activity assays (1,12). An isoelectric point protein marker was electrophoresed on either side of the apoplastic samples and stained separately for total protein using Coomassie blue stain (Fig. 5).

RESULTS AND DISCUSSION

Growth Under Space Flight Conditions

Plants from super dwarf wheat seed lot 2 had productive growth under greenhouse conditions without any disease symptoms and with normal seed set. As a precaution against microbial contamination, the seed was surface sterilized prior to use in the CHROMEX-06 spaceflight experiment. When the seedlings were examined after 8 days of growth in microgravity, approximately 50% of the initial 72 plants showed visible signs of fungal contamination. This fungal contaminant produced abundant white, cottony aerial mycelium with profuse mycelial growth on elevated roots and leaf sheaths or wheat culms. The leaves of infected plants were chlorotic, twisted and narrowed or wasted.

Seed as a Source of Fungal Contamination

The super dwarf seed (lot 2) used in the spaceflight missions had extensive internal contamination by endophytic fungi. Fungal mycelia grew out of the seed in spite of rigorous surface sterilization treatments with ethanol and sodium hypochlorite. Two fungi were observed most frequently from this lot of seeds. One was identified by its spore characteristics and its brown to black mycelium as a *Cladosporium* species (26). *Cladosporium* has also been identified as a seed-borne endophyte of wheat (20). The second fungus was identified as an *Acremonium* species based on the size and morphology of conidia and conidiogenous cells as well as colony characteristics *in vitro* (13,14,27). The small hyaline conidia produced white to slightly pink or tan mycelia and the coloration of the reverse phase on PDA plates was tan to brown near the center of the colony. This *Acremonium* species is particularly aggressive and grows rapidly in culture, producing abundant conidia.

Acremonium species are endophytic in other grasses and are seed-borne (5, 17,20, 21, 27). The fungus isolated from the leaf sheath tissue from the space-flown plants had the

same growth characteristics as the *Acremonium* isolate from wheat seed lot 2.

The *Acremonium* fungus was isolated from other super dwarf seed lots 1,3 and 4 when the seed was subjected only to surface sterilization protocols (Table 1). In collaboration with Dr. William Campbell at Utah State University, we have demonstrated that the *Acremonium* endophyte was also present in seed lot 2 used in the NASA/Mir mission.

Elimination of Seed-Borne Endophytes

Only heat treatment of the seed in addition to surface sterilization treatments eliminated seed-borne microbial contamination (Table 2). Heat treatments at 40°C followed by subsequent water washes equilibrated at 40°C slightly reduced the amount of contamination from that seed that receiving only surface sterilization. Heat treatment at 50°C with a 40°C water wash further decreased the fungal growth from the seed. Prolonged heat treatment at 50°C followed with 50°C equilibrated washes was effective in the completely eliminated endophytic fungi and did not reduce germination of the seeds.

Percent sterility and germination were assessed (Table 2). The numbers represent the means of three replications each with 100 seeds. Differences in % sterility between heat treatments in seed lots 1, 2 and 3 were significant ($p \leq 0.001$). Percent sterility as well as % germination was the highest for seed lots 4 and 5.

Table 2. Effect of heat treatment on the elimination of microbes from super dwarf wheat seed.

Seed Lot (Year)	Heat °C	Water Wash °C	% Sterility	% Germination
1	22	22	7 ± 4.0	52 ± 10.7
(1991)	40	40	17 ± 6.0	66± 5.3
	50	40	67 ± 16.0	74 ± 11.3
	50	50	100 + 0.0	80+ 5.0
2	22	22	7 ± 3.3	55 ± 10.0
(1993)	40	40	20 ± 3.3	73 ± 4.0
	50	40	70 ± 13.3	74 ± 2.6
	50	50	100 + 0.0	95+ 6.7
3	22	22	28 ± 6.0	78 ± 6.3
(1993)	40	40	42 ± 7.3	77 ± 4.0
	50	40	74 ± 9.3	81 ± 5.3
	50	50	100 + 0.0	89 + 5.3
4	22	22	70 ± 13.3	87 ± 4.0
(1995)	40	40	80 ± 6.0	80 ± 13.3
	50	40	89 ± 5.3	84 ± 5.3
	50	50	99 + 2.7	94 + 5.3
5	22	22	98 ± 4.0	86± 5.3
(1995)	40	40	99 ± 6.0	85± 3.3
	50	40	100 ± 0.0	88 ± 4.0
	50	50	100 + 0.0	87+ 14.6

Alternative sterilization procedures for the elimination of microbial endophytes from super dwarf seed were tested (Table 3) using an endophyte-infected seed (lot 4) and an endophyte-free seed (lot 5). Differences in percent sterility between the surface sterilization treatments for seed lot 4 were significant ($p \leq 0.001$). There were no apparent differences in % sterility between sterilization protocols for the microbial-free seed lot 5.

Table 3. Alternative procedures for the elimination of microbial endophytes from super dwarf seed

Treatments	Seed Lot (Year) 4 (1995) % Sterility	4 (1995) % Germination	5(1995) % Sterility	5 (1995) % Germination
None	0 ± 0.0	85 ± 5.0	80 ± 5.3	79 ± 5.0
Conc. H2SO4	0 ± 0.0	80 ± 10.3	99 ± 2.7	87 ± 5.3
30% H2O2	10 ± 20.0	82 ± 6.3	100 ± 0.0	95 ± 2.6
Sodium Hypochlorite	70 ± 13.3	87 ± 4.0	98 ± 4.0	86 ± 5.3
Sodium Hypochlorite and Heat	100 ± 0.0	87 ± 4.0	100 ± 0.0	93 ± 2.6

To test the efficacy of our methods to generate endophyte-free seed, seed from lot 2 and 3 were subjected to the specified heat treatment and wash procedure, followed by surface sterilization using sodium hypochlorite. These seedlings were grown for 8 days in prototype PGCs, identical to those used in the CHROMEX-06 spaceflight experiment under similar environmental conditions for plant growth. Plants grown from these seeds showed no symptoms of disease, which included chlorosis and wasting or twisting of leaves and there was no visible fungal growth on root, leaf sheath or leaf surfaces. Sections of root and leaf tissues from these plants were also free of any microbial contamination. The seed increased from these sterile-chamber grown plants (lot 5) was endophyte-free (Tables 2, 3) and resulted in disease-free seedlings.

Acremonium and Disease Symptoms

Super dwarf seed (lot 5) that was designated as endophyte-free produced diseased plants showing similar symptoms to infected plants in the CHROMEX-06 spaceflight experiment after inoculation with a pure spore suspension of *Acremonium*. This *Acremonium* isolate in its pathogenic growth habit exhibited aggressive growth and abundant sporulation on the outer surfaces of both leaf and root tissues, 5 days post-inoculation on its plant host.

Disease symptoms of the 8-day-old seedlings consisted of a cottony ring of mycelia girdling leaf sheaths. Leaves were chlorotic and twisted at growing points. Conidia of the fungus on leaves bearing visible mycelial growth were observed under microscopy. These conidial spores and spore-bearing hyphae or phialides resembled the structures observed from pure cultures of *Acremonium* grown on PDA plates. Control plants raised from non-inoculated lot 5 seEd lacked these symptoms.

The spread of disease symptoms originating from an endophyte-contaminated seed to seedlings from endophyte-free seeds was assessed in the enclosed PGCs housed in a prototype PGU in our laboratory. Our results indicated that just one seed contaminated with and endophytic fungus can spread secondary inoculum to other initially asceptic plants. These plants receiving secondary inoculum showed mild to severe disease symptoms by day 5 to 6 after planting (Figs. 1 and 2). Conidia were found on the outer surfaces of leaf sheaths and leaves of all plants enclosed in PGCs that contained an *Acremonium*-infected plant.

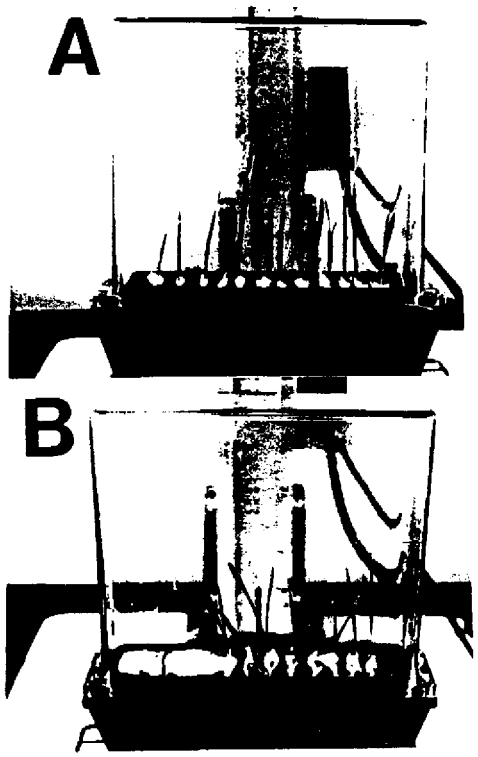

Fig. 1. Prototype plant growth chambers (PGCs) planted with 1 endophyte contaminated seed (lot 2) and 11 endophyte-free seeds (lot 5) to evaluate dispersion of fungal contamination to other healthy plants.

Fig. 2. Symptoms and disease spread caused by an *Acremonium* infected plant to other plants of super dwarf wheat in a PGC used in spaceflight experiments.

Detection of *Acremonium* in Symptomless and Diseased Plants

The size and morphology of conidiospores and fungal mycelia from pure cultures of *Acremonium* used in the inoculation of asceptic super dwarf wheat plants are shown in Fig. 3. The *Acremonium* growth was examined in both symptomless greenhouse-grown (non-inoculated) plants and in those that were grown under spaceflight conditions inoculated with *Acremonium* spores (Fig. 4).

No *Acremonium* fungal mycelia or spores was found in the heat-treated, surface-sterilized endophyte-free plants whether grown in a greenhouse or Magenta box environment (Fig. 4A). Intercellular *Acremonium* mycelia was found in the leaf sheaths in asymptomatic plants (Fig. 4B), yet no mycelia was present in root tissue and was not accompanied by sporulation (Fig. 4E). In contrast, the *Acremonium*-inoculated plants in the diseased state had abundant mycelia and conidia on the outer surfaces of both root and leaf tissues and penetrated the first several layers of the epidermal cells (Fig. 4 C,D, F).

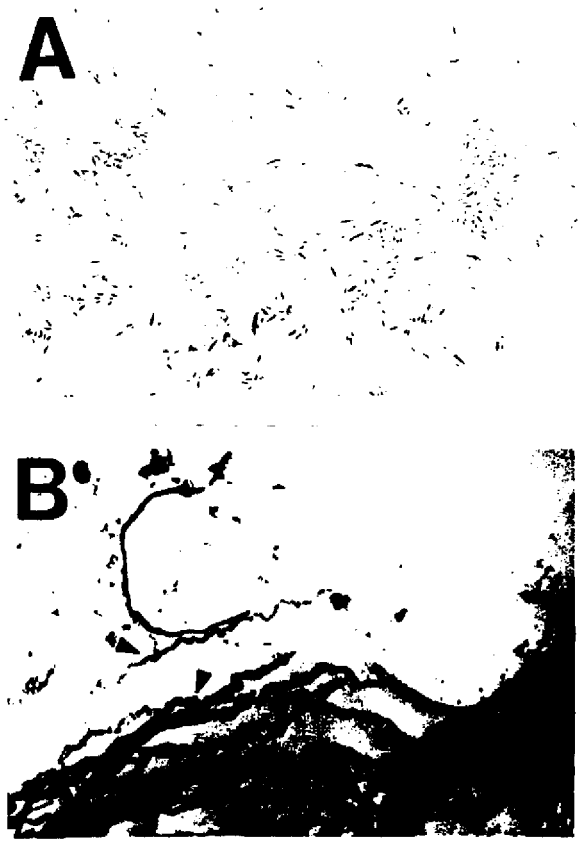

Fig. 3. Conidiospores and fungal hyphae from the *Acremonium* isolate used in inoculation studies with super dwarf wheat.

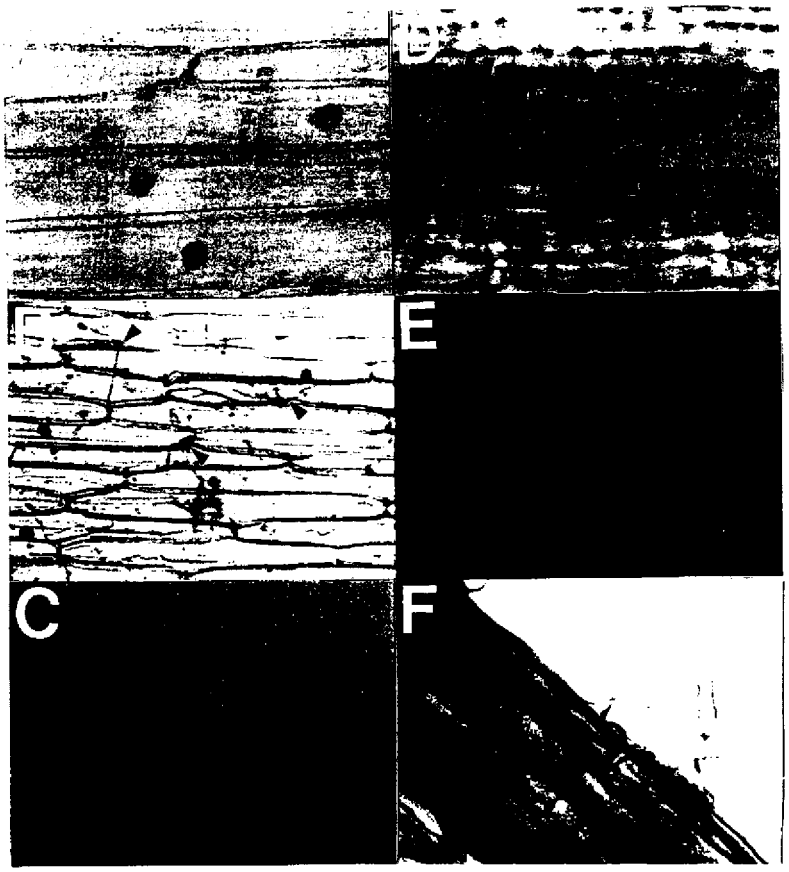

Fig. 4. Detection of the *Acremonium* fungus using an aniline-blue, lactic acid stain in symptomless greenhouse- grown and diseased plants in spaceflight environments (PGC and Magenta box).

Induction of New Isoforms of Plant Peroxidases with *Acremonium* Infection

Plant peroxidases are used in this study as predictors of plant stress to fungal attack. Total apoplastic peroxidase activity increased by approximately 60% in the leaf fractions of *Acremonium* and *Fusarium*-inoculated plants compared to non-inoculated controls. Peroxidase isozymes were induced in the leaves of *Acremonium* and *Fusarium*-inoculated plants at isoelectric points (pI) 8.0, 6.0, 5.5 and 4.6. Unique peroxidase isozymes were also found specific to the leaves from *Acremonium*-inoculated plants at pIs of 7.0 and 5.8.

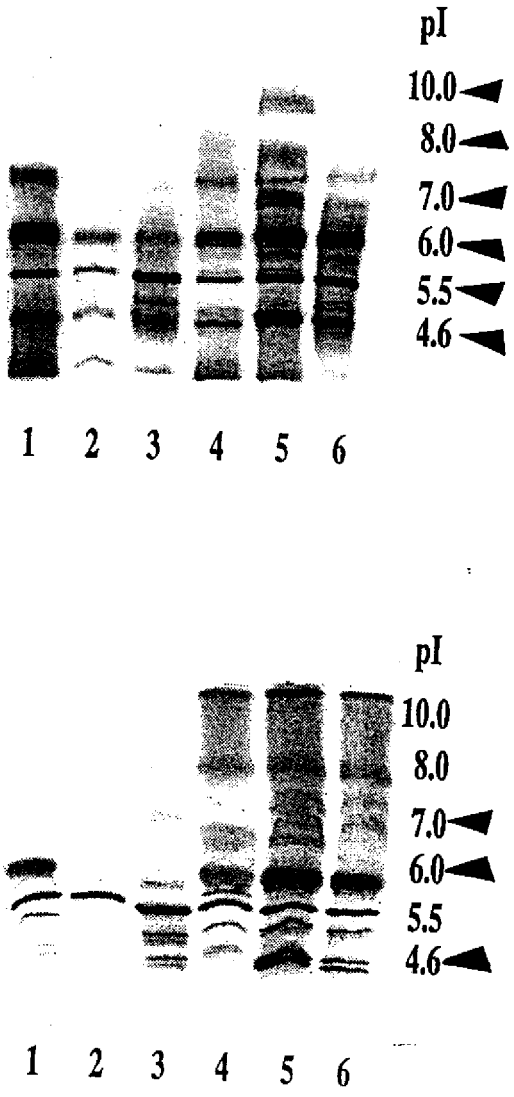

Fig. 5. Isozymic composition of apoplastic (cell wall-localized) peroxidases in roots and leaves, 8 days post inoculation with *Acremonium* and *Fusarium culmorum* detected by two separate activity stains for peroxidases: catechol/p-phenyldiamine (Fig. 5A) and chloronapthol (Fig. 5B). **Lane 1**, root apoplast from non-inoculated control; **Lane 2**, root apoplast from *Acremonium*-inoculated seeds; **Lane 3**, root apoplast from *Fusarium*-inoculated seeds; **Lane 4**, shoot apoplast (leaf sheaths and leaves) from non-inoculated control, **Lane 5**, shoot apoplast from *Acremonium*-inocualted seeds; **Lane 6**, shoot apoplast from *Fusarium*-inoculated seeds.

CONCLUSIONS

Microbiological surveys of returning spaceflight missions indicate that microbes are inevitable in space environments (4, 15, 22,23). Some of these organisms are likely to be potential plant pathogens and their pathogenicity may be enhanced by plant growth under spaceflight conditions. Our findings support the need to screen germplasms used in spaceflight research in order to determine if endophytic contamination is a problem after surface sterilization procedures have been implemented. The introduction of just one contaminated seed can result in deleterious consequences to the plant.

In order for meaningful plant growth and development experiments to be carried out in spaceflight environments, we must be alerted to the effects that endophytic fungi can have on plants grown under spaceflight conditions used in microgravity. Infection of super dwarf wheat by the *Acremonium* endophyte influenced the peroxidase isozyme pattern in the plant (3). Thus our original goal of understanding the impact of microgravity on plant peroxidases was confounded by the complexity of the plant-microbe interactions that resulted. We recommend that plants be routinely examined for microbial sterility at the end of spaceflight missions to ensure that changes are in response to microgravity and not due to complications of microbial contamination.

Strategies to control pathogens in space are essential to the development of adequate plant growth conditions aboard the International Space Station and to maintain the quality and production of food resources.

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