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# Comparative floral development of Mir-grown and ethylene-treated, earth-grown Super Dwarf wheat

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## Summary

To study plant growth in microgravity, we grew Super Dwarf wheat (*Triticum aestivum* L.) in the Svet growth chamber onboard the orbiting Russian space station, Mir, and in identical ground control units at the Institute of BioMedical Problems in Moscow, Russia. Seedling emergence was 56 % and 73 % in the two root-module compartments on Mir and 75 % and 90 % on earth. Growth was vigorous (produced ca. 1 kg dry mass), and individual plants produced 5 to 8 tillers on Mir compared with 3 to 5 on earth-grown controls. Upon harvest in space and return to earth, however, all inflorescences of the flight-grown plants were sterile.

To ascertain if Super Dwarf wheat responded to the 1.1 to 1.7 $\mu mol \cdot mol^{-1}$ atmospheric levels of ethylene measured on the Mir prior to and during flowering, plants on earth were exposed to 0, 1, 3, 10, and 20 $\mu mol \cdot mol^{-1}$ of ethylene gas and 1200 $\mu mol \cdot mol^{-1}$ $CO_2$ from 7 d after emergence to maturity. As in our Mir wheat, plant height, awn length, and the flag leaf were significantly shorter in the ethylene-exposed plants than in controls; inflorescences also exhibited 100 % sterility. Scanning-electron-microscopic (SEM) examination of florets from Mir-grown and ethylene-treated, earth-grown plants showed that development ceased prior to anthesis, and the anthers did not dehisce. Laser scanning confocal microscopic (LSCM) examination of pollen grains from Mir and ethylene-treated plants on earth exhibited zero, one, and occasionally two, but rarely three nuclei; pollen produced in the absence of ethylene was always trinucleate, the normal condition. The scarcity of trinucleate pollen, abrupt cessation of floret development prior to anthesis, and excess tillering in wheat plants on Mir and in ethylene-containing atmospheres on earth build a strong case for the ethylene on Mir as the agent for the induced male sterility and other symptoms, rather than microgravity.

**Key words:** ethylene – microgravity – Mir – pollen – seed set – sterility – Super Dwarf – Svet – Triticum aestivum – wheat

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## Introduction

Plants are known to be extremely sensitive to gravity. A young plant stem that is growing vertically, for example, when placed on its side or tipped only a few degrees from the vertical, will right itself within a few hours (e.g., Salisbury 1993). Thus, it is of great interest to study plant growth in microgravity. If plants can be grown through a complete life cycle (i.e., from seed-to-seed) in microgravity, we can conclude that there is no physical, chemical, or biological process that is so dependent on gravity that plants cannot mature in its absence. It has long been a goal of researchers in the field of plant gravitropism, as well as space biologists, to see if, in the microgravity of an orbiting space vehicle, it is possible to grow plants that are comparable in every important way with plants grown on earth under identical environmental conditions except for gravity.

There is also a practical reason to grow highly productive food plants in space: Such crops might supply oxygen, food, and water and might remove $CO_2$ and nitrogenous wastes produced by human occupants of the space craft. Crops used this way would be part of a *bioregenerative life-support system* (BLSS). Such a BLSS could be critical to mankind's long-term presence in space or reduced gravity environments as on the Moon or Mars (Dutcher et al. 1994, Halstead and Dutcher 1987, Kuang et al. 1995, Salisbury 1999).

Wheat has been shown to produce extremely high yields in controlled environments – nearly six times the world record yields in the field (Bugbee and Salisbury 1988, 1989). During 1995 and 1996 in a joint United States/Russian project, we grew a short cultivar of wheat, called «Super Dwarf,» in the Russian space station Mir (Bingham et al. 1995, 1997, Salisbury 1997). Mechanical problems plagued us in 1995, but these were remedied, and we completed a 123-day experiment in 1996 as well as a 39-day experiment in 1996/97. The plants grew extremely well, producing more plant biomass than in any previous space experiment (thanks to higher irradiance than in previous experiments), and in the 123-day experiment, there were ca. 280 wheat heads produced. When the plants were returned to earth, however, all the heads proved to be sterile. Not a single seed was produced.

In our post-mortem discussions, we listed about a dozen reasons why seeds might not have formed. Principal among these were high $CO_2$ water-logged substrate, anoxia, ethylene, and microgravity. Because density-driven convection does not occur in microgravity, Kuang et al. (1993) suggested that a high boundary layer of humidity might have caused the failure of anthers to dehisce, or oxygen deprivation might have impacted the anthers. Indeed, Kuang et al. (1995) observed that after 6 d in spaceflight onboard the space shuttle <Endeavour> (STS-54), *A. thaliana* exhibited collapsed ovaries with empty ovules and deformed empty microspores. Ethylene was not present in the post flight analysis of gas in each chamber. Instead, Kuang et al. (1995) attributed the problem to $O_2$ depletion in the boundary layer; they demonstrated a solution by stirring the air in the chamber during a later experiment, called Chromax 5. Murgia et al. (1992) have shown that pollen viability is severely decreased by anoxia. Also, Quebedeaux and Hardy (1973) observed that a 5 % oxygen atmosphere completely inhibited seed development in soybean.

None of these suggestions applies to our experiment, however, because air was never stagnant in Mir's growth chamber (called Svet); fans pulled it over the plants and past the lamps. And with the lights on, those green plants were probably producing oxygen that kept their boundary layers well supplied.

Another possible cause of sterility is root-zone anoxia, which might have occurred for short periods during programmed water-status changes (Bingham et al. 1996 a, b, 1997, Yendler et al. 1996), mimicking the low atmospheric oxygen content observed in flooded soils on earth (Thomson and Greenway 1991). This seems unlikely, however, because root-zone anoxia, if it occurred, was sporadic, whereas wheat heads that formed over a six-week period were *all* sterile.

Wheat plants grown onboard Mir were exposed to high levels of $CO_2$ (4,000 to 10,000 $\mu mol \cdot mol^{-1}$, continuously monitored by our infrared gas analyzer), which were super-optimal and shown to reduce yield in wheat (Grotenhuis and Bugbee 1997, Jiang et al. 1998, Salisbury 1997, Voesenek et al. 1997). Bugbee et al. (1994) and Grotenhuis and Bugbee (1997) observed that elevating $CO_2$ levels from 350 to 1,200 $\mu mol \cdot mol^{-1}$ increased the seed yield of wheat and rice, *Oryza sativa* L., by 30 to 40 %, but $CO_2$ enrichment of 2,500 to 20,000 $\mu mol \cdot mol^{-1}$ reduced seed yield 25 to 35 % although it did not affect vegetative growth. Additionally, elevated $CO_2$ levels of 1,000 to 10,000 $\mu mol\ mol^{-1}$ increased ethylene synthesis in some plants (Bugbee et al. 1994). Even at 10,000 $\mu mol \cdot mol^{-1}$ $CO_2$ however, plants were never completely sterile.

Of the possible causes of our wheat sterility, only two seemed to have a high probability of actually being causal: microgravity and the presence of ethylene in the Mir atmosphere. Ethylene has long been known to cause male sterility and other effects in cereals (e.g., Abeles et al. 1992, Bennett and Hughes 1972, Keyes and Sorrells 1990, Rowell and Miller 1971, Voesenek et al. 1997). In one study, ethylene generated from ethephon (2-chloroethylphosphonic acid) spray applications induced abortion of sporogenous cells at both pre- and postmeiotic stages of microsporogenesis in barley (Colhoun and Steer 1983).

It is known that plants produce ethylene in response to abiotic and biotic stresses throughout their development and that ethylene levels even below 1 $\mu mol \cdot mol^{-1}$ (equivalent to ppm by volume: ethylene $\cdot$ $air^{-1}$) will induce adverse biological activity (Abeles et al. 1992, Voesenek et al. 1997). Moreover, wheat plants have been shown to produce ethylene during vegetative growth (Petruzzelli et al. 1994) and grain filling (Beltrano et al. 1994, Labrana et al. 1991). Finally, various species of fungi are known to produce ethylene (see references in Abeles et al. 1992), and over 100 species of fungi were identified in the Mir Space Station (L. Chernova, IMBP, Moscow, Russia, personal communication, 1997). A white fungus grew at the base of our Super Dwarf wheat plants although it did not appear to be directly harmful.

Although the Mir atmosphere had not previously been analyzed for ethylene, thirteen gas samples had been collected during our 1996 experiment, and when these were re-analyzed they were found to contain from 1.1 to 1.7 $\mu mol \cdot mol^{-1}$ ethylene with levels of about 1.4 $\mu mol \cdot mol^{-1}$ ethylene consistently during the anthesis period (Bingham et al. 1996 a, James et al. 1998). Thus, we established the hypothesis that the observed sterility was caused by ethylene and not by microgravity. Three ways to test this hypothesis were apparent:

1. Add ethylene scrubbers to the equipment used in Mir to grow wheat, and then repeat the experiment. This is certainly the most obvious test, but neither NASA nor the Russian Space Agency would support such an experiment. (It was said to be too costly to develop the necessary equipment at that time.) Such an experiment will surely be carried out in future space studies.
2. Use a wheat cultivar that is more resistant to ethylene in the existing equipment. This has been done successfully with Apogee, a cultivar developed specifically for space studies and thus short enough to grow in Svet on Mir. Recently, Klassen and Bugbee (2000) have shown that Apogee is less sensitive to ethylene than the Super Dwarf wheat cultivar. All Super Dwarf plants receiving 1 $\mu mol \cdot mol^{-1}$ ethylene or higher were 100 % sterile, while there was some Apogee seed set at that level of ethylene. Probably because of the ethylene in Mir, only a few seeds of Apogee were produced, but these were carried through a second generation; that is, seed-to-see-to-seed (Levinskikh et al. 1999 a). Details will be reported elsewhere.
3. See if Super Dwarf wheat, grown under ethylene concentrations comparable to those in the Mir atmosphere during our 1996 experiment, exhibits identical symptoms to those observed in our Mir wheat, which in addition to the sterility included shortened leaves and stems, excessive tillering, and various microscopic features of the wheat heads. This is the approach described in this paper.

In spite of the known effects of ethylene on cereals, it was necessary to carry out these experiments because, of the studies on cereal sterility known to us (e.g., Bennett and Hughes 1972, Foster et al. 1991, Keyes and Sorrells 1990, Rowell and Miller 1971, and Taylor et al. 1991), all but one used ethephon (2-chloroethylphosphonic acid; Ethrel) in solution applied to their test plants as the source of ethylene, making it impossible to compare their results with our own. The one exception (Reid and Watson 1985) grew oats (*Avena sativa* L.) and not wheat; ethylene reduced the number of florets/plant from 464 in the zero ethylene control to only two in the 0.150 $\mu mol \cdot mol^{-1}$ ethylene treatment. To our knowledge, the results reported here are the first to test a series of low ethylene concentrations on growth and reproduction of wheat, especially at high $CO_2$ concentrations.

Although our 1996/97 experiments in Mir were the first to produce quantities of plant biomass in microgravity comparable to those produced in ground controls, there is a relatively long history of plant experiments in space. Scientists in the former Soviet Union were able to grow plants for several months on Salyut-4, 6 and 7 (Dubinin et al. 1977, Gorkin et al. 1980, Kodyum et al. 1983, Merkys et al. 1981, Merkys and Laurinavichyus 1983) and in their orbital space station Mir (Mashinsky et al. 1994, Nechitailo and Mashinsky 1993). Working with carrot (*Daucus carota* L.), cucumber (*Cucumis sativus* L.), dill (*Anethum graveolens* L.), onion (*Allium cepa* L.), pea (*Pisum sativum* L.), radish (*Raphanus sativus* L.), and wheat (*Triticum aestivum* L.) onboard various spaceflight vehicles, the Soviets obtained fair vegetative growth from germinated seeds, but plant death occurred routinely at or before the flowering stage. Moreover, numerous long-term plant experiments in space failed to produce normal growth (vegetative biomass or seed yield) compared with those grown on earth (Dutcher et al. 1994, Halstead and Dutcher 1987, Mashinsky et al. 1988, Merkys and Laurinavichyus 1983, Nechitailo and Mashinsky 1993).

After many attempts, however, Merkys and Laurinavichyus (1983) were successful in growing *Arabidopsis thaliana* (L.) Heynh. from seed-to-seed in Salyut-7. Yet the seeds produced in space were shrunken and exhibited a 38 % reduction in germination compared to seeds from earth-grown control plants. Cowles et al. (1984) also reported reduced vigor of oats (*Avena sativa* L.), mung bean (*Vigna radiata* (L.) Wilczek.), and pine (*Pinus elliotti* Engelm.) seedlings on the shuttle Orbiter STS-3. Conger et al. (1998) also observed that 11 days on shuttle Orbiter STS-64 dramatically suppressed the initiation and development of somatic embryos from mesophyll cells of orchard grass (*Dactylis glomerata* L.) grown *in vitro*, whereas normal somatic embryos were initiated and developed from similarly cultured leaf segments in earth-grown controls. Krikorian and O'Connor (1984) observed chromosome breakage and bridge formation in space-grown oat and sunflower (*Helianthus annuus* L.) seedlings.

More recently, the reports of Kuang et al. (1996) are encouraging as they observed normal development of seeds in *Arabidopsis thaliana* plants that flowered during 11 days in space on shuttle Orbiter STS-68. Also, Krikorian et al. (1981) flew totipotent cells and cell clusters of carrot onboard Cosmos 782 and 1129 Biosatellites. They observed that carrot embryos developed into well-defined plants with no detectable differences between space- and earth-grown plantlets.

Mashinsky et al. (1994) achieved the formation of a single spike on Super Dwarf wheat grown in a small cylinder (called Svetoblock-M) onboard the Mir, but it remained seedless. Upon return to earth, tillers that formed while onboard Mir matured and produced seed when placed under higher light intensity and ambient $CO_2$. The experimenters did not ascribe these poor responses to microgravity, but to environmental and micro-environmental factors on Mir: low photosynthetic photon flux, short photoperiods, high to toxic levels of $CO_2$ (typical for virtually all space experiments), inadequate water distribution in the rooting medium, or perhaps some unknown factor(s) that delayed the flowering process. Mashinsky et al. (1994) suggested that wheat was capable of forming reproductive organs in spaceflight provided conditions similar to those required for plant growth on earth were met.

To reiterate, the objectives of our earth-grown Super Dwarf wheat experiments were to establish a dose-response curve of Super Dwarf wheat to low levels of ethylene gas in the presence of elevated $CO_2$, observing vegetative growth and floral development. We present here the comparative floret development of Mir-grown and ethylene-treated, earth-grown wheat plants with analyses of the cause(s) of Super Dwarf wheat sterility.

## Materials and Methods

### Flight experiment: Super Dwarf wheat plants growing conditions on Mir

The Svet (means light in Russian) growth chamber onboard the Russian space station Mir (means peace in Russian) has 0.1 $m^2$ of growing area, and accommodates plants up to 40 cm tall (Bingham et al. 1995, Salisbury 1997). The Svet was designed by Russian (Institute of BioMedical Problems: IMBP) and Bulgarian scientists and built at the Space Research Institute, Sofia, Bulgaria under the supervision of Tania Ivanova. Ground-based controls at IMBP in Moscow were grown in a Svet unit identical to the one on Mir. Fluorescent lamps (Osram Delux S, 11 W, Sylvania cool white, Branch of Siemens Co., Danvers, MA) provided light at a photosynthetic photon flux (*PPF* = micromoles of photons between 400 and 700 nm per square meter second) of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with 18 to 23 h of light and 1 to 6 h darkness and temperature regimes of ca. 27/21 °C (day/night). An environmental control system capable of measuring several parameters including $CO_2$ was built at Utah State University (Space Dynamics Laboratory) and installed in Svet. Detailed description of the hardware can be found in Bingham et al. (1995, 1996 a, b).

The root module contained two compartments, each 31.9 cm long, 17.5 cm wide, and 9.0 cm deep (Salisbury 1997). These were filled with a plant-nutrient-loaded clinoptileolite (Cp) zeolite (1–2 mm diameter particles) called Balkanine (supplied by Tania Ivanova). The zeolite is a natural hydrated aluminosilicate mineral with a high degree of internal tunneling and cation exchange capacity (Boettinger and Graham 1995, Ming and Mumpton 1989). Twenty-six seeds of wheat (*Triticum aestivum* L. cv. Super Dwarf: CIMMYT selection CMH79.481–1Y8B-2Y-2B-OY; Salisbury et al. 1998), previously attached to plastic strips with a water-soluble white glue (Levinskikh et al. 1999 a, b) were placed 1 cm below the surface between two cotton-fabric wicks in each of the two rows (26 seeds/row) of each root compartment for a total of 104 seeds. Water was injected through hydroaccumulators embedded in the Balkanine medium in each of the two compartments and transferred by capillary action through the wicks directly to the seeds and the surrounding Balkanine. Water quantity and distribution were measured hourly by 16 moisture sensors (two rows of four each in each compartment, placed at 4.0 and 7.0 cm in the substrate). Approximately 65 % relative water content was maintained in each of the root compartments (Bingham et al. 1996 a, b, Salisbury 1997, Yendler et al. 1996).

### Flight experiment: harvest, preparation, and examination of plant material from Mir

Wheat plants, both ground controls and on Mir, were sampled at 21, 39, 52, and 63 d and harvested at 123 d from emergence. Fresh samples were stored in plastic bags containing aqueous fixative (40 $g \cdot L^{-1}$ formaldehyde, 10 $g \cdot L^{-1}$ glutaraldehyde, buffered with 0.1 $mol \cdot L^{-1}$ cacodylate at pH 7.2; McDowell and Trump 1976). Dry samples were stored in «Sorb-it-Silica™» (Silica Gel, Fisher Scientific, Pittsburgh, PA). Upon return to earth, all Mir- and earth-grown spikes were examined over a narrow slit of light (makes seeds visible) to monitor for seeds. Mir-grown spikes showed 100 % sterility compared with relatively good seed set on earth-grown spikes (Levinskikh et al. 2000).

### Dose-response experiment: plant growing conditions on earth, ethylene treatment

Super Dwarf wheat seeds were planted into 0.071 $m^2$ hydroponic plastic flats and covered with 2 mm diameter extruded diatomaceous earth (Isolite, Sundine Enterprises, Arvada, CO). Plastic flats were placed in six plexiglass hydroponic cylinders (30 cm dia., 50 cm tall; total volume = 35 L) in a controlled-environment chamber (Percival, Model PT-80, Boone, IA) at 23 ± 2 °C day/night. Air flow to each cylinder was maintained at 20 $L \cdot min^{-1}$ and regulated by large rotameters (Model RMA, Dywer Instruments Inc., Michigan City, IN). Fans in the top of each cylinder mixed the internal air and maintained relative humidity at about 80 %. Oxygen in the nutrient medium, which remained constant at 85 % of saturation or greater, was replenished as it cascaded back into the reservoir. Atmospheric pressure at Logan, UT (1400 m elevation) was 86 ± 1 kPa (85 % of sea level). Carbon dioxide level was maintained at 1200 $\mu mol \cdot mol^{-1}$ (micromoles of $CO_2$ per mole of air) throughout the study (Grotenhuis and Bugbee 1997). Pure $CO_2$ and ethylene gases were mixed with filtered air pumped from outside the building, and plants' exposure to ethylene gas dilutions of 0, 1, 3, 10, or 20 $\mu mol \cdot mol^{-1}$ began 7 d after emergence. Recirculating nutrient solutions specifically developed for wheat were pumped into each cylinder from the reservoir by a magnetic-drive pump at a rate of 0.08 $L \cdot s^{-1}$ (Grotenhuis and Bugbee 1997). Gas levels of each plant cylinder were controlled by small rotameters (Model RMA, Dywer Instruments Inc.) and monitored by gas chromatography.

Plant exposure to cool-white light (*PPF* between 32.4 and 36 $mol \cdot m^{-2} \cdot d^{-1}$, given at 450–500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) began 10 d after seeding with a daily 12 h photoperiod. This day was appointed as the emergence date as it was the plants' first exposure to light. Plants were thinned to ca. 30 plants per cylinder at emergence. Fourteen days after planting, the daily photoperiod was increased to a 20/4 d/n regime. Mylar skirts were placed around the cylinders and maintained at the same height as the plants to minimize the influence of side lighting and to imitate canopy conditions noted in the center of field plots.

**Table 1.** Size of anthers and pollen grains in standard wheat varieties compared to Mir-grown and ethylene-treated, earth-grown Super Dwarf wheat.

| | Anther | | | Pollen grain | References |
|---|---|---|---|---|---|
| | Ln (mm) | Width (mm) | Size ($mm^2$) | Dia. ($\mu m$) | |
| Std. Wheat | 3.19–4.41 | 0.57–1.07 | 1.82–4.72 | 47.1–52.7 | Kherde et al. 1967 |
| Chinese Sp. | 3.45 | – | – | – | De Vries 1971 |
| Super Dwarf | | | | | |
| Grnhse | 1.53±0.05 | 0.41±0.01 | 0.63±0.03 | 46.1±1.6 | |
| Mir | 1.38±0.13 | 0.33±0.02 | 0.46±0.03 | 41.8±1.4 | |
| $C_2H_4$ | | | | | |
| NASA/ARC | | | | | |
| 2 $\mu mol \cdot mol^{-1}$ | – | – | – | 33.8±0.6 | |
| USU | | | | | |
| Control | | | | 43.0±0.05 | |
| 1 $\mu mol \cdot mol^{-1}$ | | | | 38.4±0.08 | |
| 3 $\mu mol \cdot mol^{-1}$ | | | | 37.9±0.09 | |
| 10 $\mu mol \cdot mol^{-1}$ | | | | 36.1±0.08 | |
| 20 $\mu mol \cdot mol^{-1}$ | | | | 35.6±0.25 | |

### Dose-response experiment: harvest, preparation, and examination of ethylene-treated plants on earth

Plants were sampled at 63, 77, 85, and 88 d after emergence for morphological and SEM and LSCM studies of developmental stages of ontogeny. Morphological data measured included plant height (mm), number of tillers, number of leaves per tiller, spike length (mm), awn length (mm), internode length (mm), number of spikelets per spike, number of nodes per rachis, number of florets per spikelet, and percent seed set. The experiment concluded when the control plants reached physiological maturity at 88 days after emergence, determined by the loss of green color in the seeds.

### Scanning electron microscopy (SEM) procedures

Wheat flowers consisting of pistils, stamens, and lodicules were excised from the fixed spikelets, dehydrated with graded series of ethyl alcohol, acetone, and tetramethylsilane (TMS), and evaporated overnight at 25 °C. The processed floral tissues from Mir and the ground controls were mounted onto circular aluminum stubs (15 mm dia) with an inert glue, «Torr Seal™» (Ted Pella, Inc., 3595 Mountain Lakes Blvd., Redding, CA). To enhance the contrast and density of the tissues, between 12.6 and 21.0 nm of gold (Au) and palladium (Pd) (60 % : 40 %) were evaporatively applied to the tissue surfaces with an Ion Beam Sputterer (Model IBS/TM200S, VCR Group, Inc.). The ion gun produced 5 kV and 2 mA. Specimens were examined and photographed with a scanning electron microscope (Hitichi S-4000) at an accelerating potential between 2 and 5 kV.

### Laser scanning confocal microscopy (LSCM) procedures

Anthers were crushed to expose pollen grains and incubated in 250 µL propidium iodide in 50 % ETOH and 100 µL of dimethyl sulfoxide (DMSO) for one hour in darkness in 2 mL microfuge tubes. After centrifugation for 5 min at 1200 rpm, the supernatant was decanted, and a portion of the pellet was placed in 100 µL of Prolong Antifade Kit (Molecular Probes, Eugene, OR). Slides were cover-slipped and sealed with cyanoacrylate glue to minimize oxidation of the dye. The BioRad MRC-1024 LSCM was equipped with an Argon–Krypton (Ar–Kr) laser. Pollen grains were optically sectioned and examined with the LSCM interfaced with a Nikon Eclipse TE-300-inverted microscope. By raising or lowering the sample in discreet steps, thin optical fluorescent sections were generated and with the appropriate computer software were combined into a composite image. The Ar-Kr laser produces light centered at 488 and 568 nm, with the generated wavelengths overlapping at 536 nm. The light at 536 nm caused the propidium iodide in the pollen nuclei to fluoresce, generating light centered at 617 nm.

## Results and Discussion

### Flight experiment: observations on Mir-grown Super Dwarf wheat plants

Emergence percentages recorded from seeds in compartments one and two of the root module 7 d after planting were 56 % and 73 % on Mir and 75 % and 90 % on earth. Seedling growth was vigorous until the onset of anthesis, at which time the main spike and primary tiller spikes began to senesce, and new tillers with spikes were formed producing 5–8 tillers per plant on Mir compared with 3–5 on ground controls. Video tapes (14 total) were taken of plants on Mir and transmitted to earth at intervals. These videos showed what appeared to be normal ontological stages of development with the largest biomass of wheat plants (280 spikes harvested) ever produced in microgravity. Although we only received fixed or mature, dry plants, and almost half of the plants were removed for sampling, we estimate that the fresh mass would have exceeded a kilogram if all plants had been allowed to mature. Because of the repeated tillering, anthesis continued

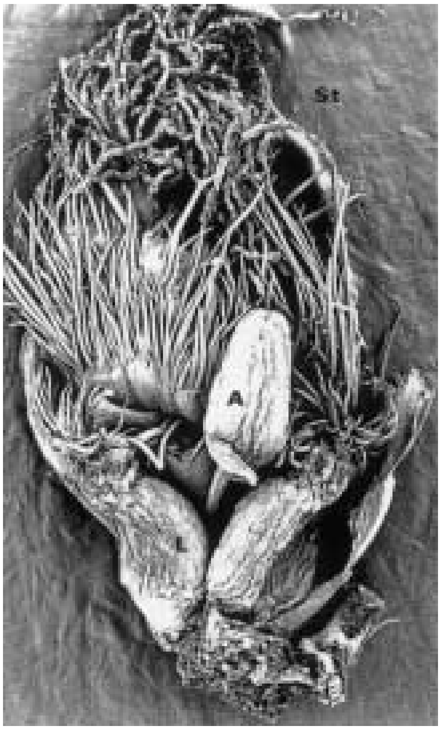


**Figure 1.** SEM of floret harvested from a Super Dwarf inflorescence 63 d after emergence in Svet onboard Mir and stored in chemical fixative. The stigma (ST) has not yet unfurled. Stamens, pistil, and the enlarged lodicules (L) indicate that the floret is in the pre-anthesis stage. The Anther (A) appears normal. (Anther length: ca. 1.4 mm; see Table 1.)

**Figure 2.** Super Dwarf wheat floret harvested from earth-grown controls. Stamens, pistil, and enlarged lodicules indicate that this floret was at the onset of anthesis. The feathery stigma of the ovary has unfurled. Anthers have started to dehisce, but pollen grains were not yet evident on the stigmatic surfaces. (Anther length: ca. 1.5 mm; see Table 1.)

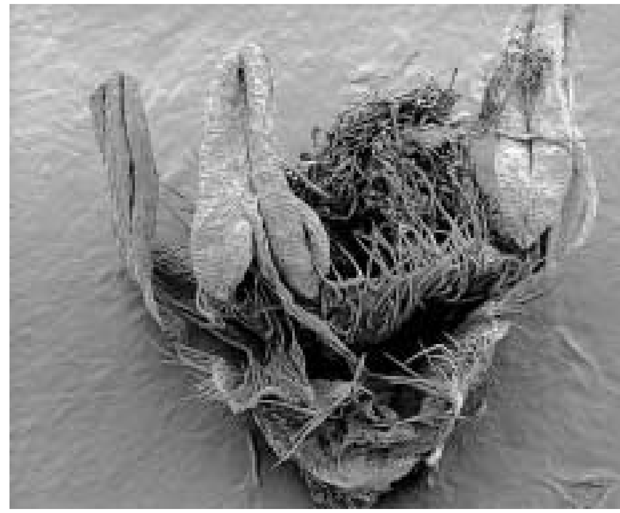

**Figure 3.** Scanning electron micrograph of stamens and pistil excised from a wheat plant grown in Svet onboard the Mir Space Station. Note the dry, withered and shrunken conditions of the Stamens, pistil and lodicules.

for approximately 40 d. Plants were all sterile upon harvest and return to earth. No pollen grains were evident on the stigmatic surfaces of Mir ovules in contrast to the commonly observed pollen-covered stigma of developing ground plants. Measurements of wheat anthers from SEM micrographs indicated that anthers developed on ground-control plants were 10 % longer and 20 % wider than those from Mir (Table 1). Also, pollen grain diameters were 9 % greater in controls than those from Mir-grown plants.

In Mir-grown plants, development of the primary and tiller stems produced normal-looking spikes over a six-week period. Examination of Mir-grown florets (Fig. 1) containing pistils, stamens, and lodicules indicated that, although they were delayed in development, they were developing similarly to those produced in ground-based growth chambers (Fig. 2). The swollen lodicules and the firm pistil and stamens suggests that the florets were approaching or were at the onset of anthesis. All florets from Mir that were examined, however, indicated a cessation of development in about the same stage of ontogeny; that is, prior to anthesis, at which time the floret began to senesce, the anthers did not dehisce, and pollen grains began to collapse (Fig. 3). The pistil, stamens, and lodicules from a floret harvested 123 d after emergence clearly showed that they ceased development and began to senesce prior to anthesis; all of the organs appeared to have collapsed (Fig. 1–3). Manually or accidentally ruptured anthers were observed to contain an abundance of pollen grains (Figs. 4 and 5), and the exine surfaces of Mir-grown pollen grains were similar to those produced on the ground. Pollen grains from ground controls harvested at full maturity showed only minor shrunken grains (Fig. 5).

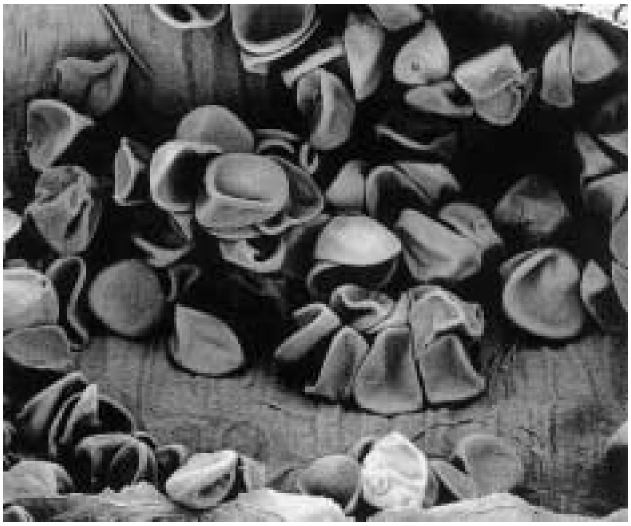

**Figure 4.** A manually ruptured anther showing senesced, collapsed pollen grains from Mir-grown wheat harvested at 63 d after emergence. (Pollen diameters: ca. 42 µm; see Table 1.)

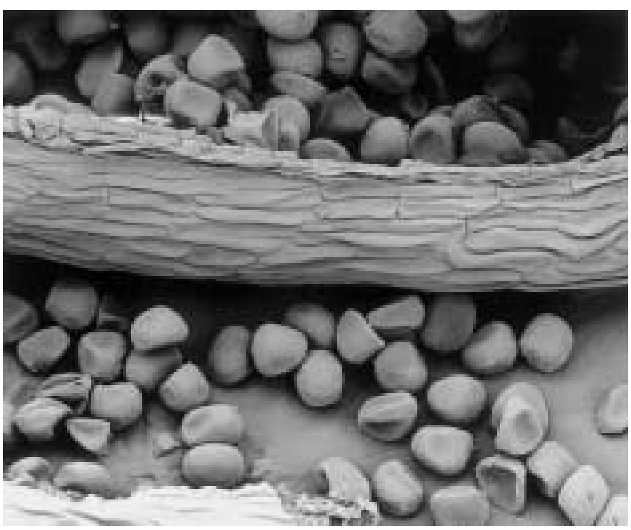

**Figure 5.** A ruptured anther showing senesced pollen grains from earth-grown wheat harvested 63 d after emergence. Note the number of pollen grains still in a firm, circular shape. (Pollen diameters: ca. 46 µm; see Table 1.)

## Dose-response experiment: observations on ethylene-treated, earth-grown Super Dwarf wheat plants

As noted in the introduction, it is well known that ethylene gas (nearly always supplied as ethephon) can cause male sterility in cereals, and measurements of samples of Mir atmosphere indicated the presence of ethylene at levels of 1.1 to 1.7 µmol · $mol^{-1}$, consistently about 1.4 µmol · $mol^{-1}$ during the anthesis period (Bingham et al. 1996 a, James et al. 1998). Thus we directed our efforts at studying responses of Super Dwarf wheat to various concentrations of gaseous ethylene. (Some members of our group, working with others, have now extended these studies to ethylene concentrations as low as 0.050 µmol · $mol^{-1}$; the results will be published elsewhere.)

To confirm that the Super Dwarf wheat cultivar was sensitive to the level of ethylene measured onboard the Mir, we exposed plants to 0, 1, 3, 10, and 20 µmol · $mol^{-1}$ of ethylene gas and 1200 µmol · $mol^{-1}$ $CO_2$. There were significant reduc-

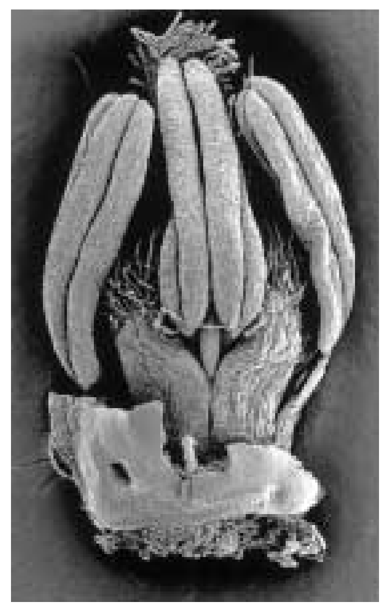

**Figure 6.** SEM of an earth-grown wheat floret at anthesis. This floret had been exposed to 20 µmol · $mol^{-1}$ ethylene for 81 d. The large bodies below the middle anther are lodicules, which have shrunk slightly. (Anther lengths: ca. 1.3 mm.)

**Table 2.** Morphometric responses of earth-grown Super Dwarf wheat plants at elevated $CO_2$ (1200 µmol $mol^{-1}$) and varying levels of ethylene gas – Logan, UT.

| Ethylene Level µmol · $mol^{-1}$ | Plant Height (mm) | No. of Tillers | Spike Ln (mm) | Awn Ln (mm) | No. Rachis Nodes | Florets/ Spikelet | Percent Fertility |
|---|---|---|---|---|---|---|---|
| 0 | 391 ± 8.2 | 5.6 ± 1.3 | 57.9 ± 1.5 | 50.9 ± 2.0 | 17.2 ± 0.4 | 3.9 ± 0.1 | 53.0 ± 3.5 |
| 1 | 275 ± 7.3 | 8.0 ± 0.7 | 44.2 ± 1.7 | 8.8 ± 1.1 | 17.2 ± 1.1 | 10.1 ± 1.1 | 0.0 |
| 3 | 251 ± 4.7 | 12.6 ± 2.2 | 40.4 ± 1.9 | 6.7 ± 0.2 | 16.8 ± 0.5 | 12.5 ± 1.1 | 0.0 |
| 10 | 221 ± 13.4 | 15.4 ± 5.2 | 40.0 ± 3.2 | 6.6 ± 0.5 | 16.2 ± 0.4 | 11.1 ± 1.3 | 0.0 |
| 20 | 199 ± 5.5 | 15.2 ± 3.2 | 43.8 ± 0.6 | 9.3 ± 1.4 | 15.8 ± 1.0 | 11.5 ± 1.7 | 0.0 |

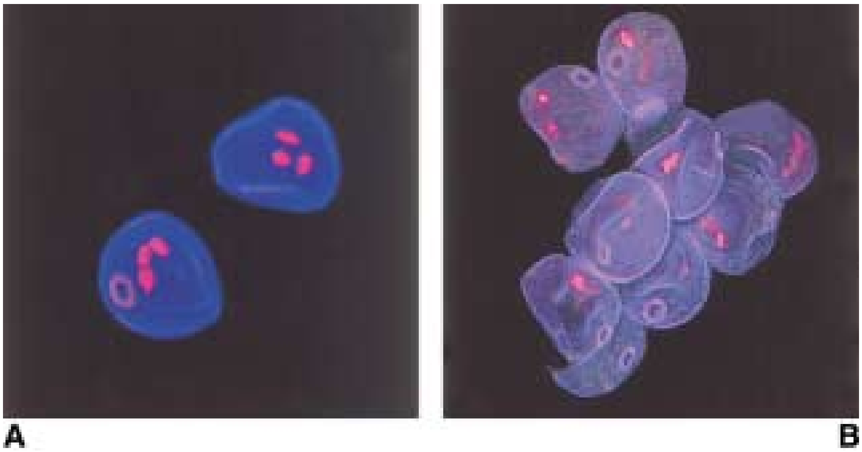


**Figure 7.** A. LSCM of pollen grains from control Super Dwarf wheat showing three propidium-iodide-stained nuclei. B. LSCM of pollen grains of Super Dwarf wheat exposed to 1 µmol · $mol^{-1}$ ethylene. Note that some pollen grains have only one or two nuclei.

tions in plant height, spike length, awn length, number of rachis nodes on the spike, and percent fertility, whereas number of tillers and number of florets per spikelet increased significantly with increased ethylene levels (Table 2). Spike and tiller awn lengths, number of rachis nodes, and number of florets per spikelet decreased with increased levels of ethylene (data not shown). Ethylene was consistent in inducing epinasty in leaves of primary and tiller plants (a length-wise curling into a cylinder). All the ethylene-treated plants in this experiment exhibited 100 % sterility. SEM examination of florets showed that their development proceeded normally but ceased prior to anthesis, and the anthers did not dehisce, thus mimicking Mir-grown wheat (Fig. 6). LSCM examination of pollen grains exposed to 1 µmol · $mol^{-1}$ ethylene or higher exhibited zero, one, and occasionally two, but rarely three nuclei, whereas those from earth-grown control plants were normally trinucleate (Fig. 7). At 10 and 20 µmol · $mol^{-1}$ ethylene, 90 – 95 % of the pollen grains did not contain any nuclei. An examination of 8 to 10 µm paraffin serial sections with a light microscope (Moscow State University) also indicated that pollen grains in the Mir-grown wheat had one or two, but rarely three nuclei as compared to the trinucleate stage observed in fertile earth-grown florets (Levinskikh et al. 1999 a, b, 2000, Veselova et al. 1999).

As noted, both anthers and pollen were smaller in ethylene-treated plants than in control plants (Table 1). Bennett and Hughes (1972) reported similar results although they used ethephon instead of gaseous ethylene. They noted that in florets in which male sterility had been induced, anther development was abnormal. Also, anthers from treated plants were smaller in size, and extrusion and dehiscence often failed compared with normal anthers. On rare occasions when dehiscence did occur, sterile pollen was released. Pollen grains did not contain any starch grains or elongated sperm nuclei, and sometimes more than three nuclei were observed. We have seldom observed three nuclei in pollen grains produced on the Mir or in ethylene-treated plants and never more than three.

The development of primary and numerous tiller spikes of wheat occurred in all of the ethylene treatments, but anthers that formed on the inflorescences did not dehisce nor did they have any fertile pollen. Flowering in wheat as in other seed-producing plants consists of a number of complex, sequential stages of ontogeny that involve pollen and embryo sac development, pollination, fertilization, and embryogenesis. Ethylene gas interrupts a crucial stage of reproductive development; namely, pollen development and anther dehiscence, thus causing a failure of seed set as reported earlier (Bennett and Hughes 1972, Reid and Watson 1985, Rowell and Miller 1971, Taylor et al. 1991).

## Evidence supporting ethylene as the cause of wheat sterility onboard Mir

Ethylene is a known potent inhibitor of seed set in wheat and other cereals by inducing male sterility. Ethylene also promotes tillering, reduces stem growth, and causes leaf epinasty. We have observed all these characteristics in Mir-grown wheat.

Although the cellular processes that regulate anther-cell differentiation and pollen release are not known, the sequential events leading to viable pollen begin with meiosis and proceed in a precise chronological manner (Goldberg et al. 1993). There are, however, many sites along the developmental pathway in which disruptions may occur. The presence of pollen that appeared to be normal suggests that the initial phases of microsporogenesis occurred in the wheat plants grown onboard the Mir Space Station, but the anthers did not dehisce. Expansion of the ovary in Mir-grown wheat florets suggests that megasporogenesis was initiated as well as the swelling of the lodicules preparatory to anthesis (Leighty and Sando 1924).

Based on Super Dwarf wheat responses in this study, levels of ethylene and $CO_2$ onboard Mir were critical during the plants' development. Thus, future flight experiments with crop plants must consider scrubbers to lower or remove ethylene, which interferes with normal anther development and dehiscence and causes abnormal nucleation of pollen grains leading to male sterility. $CO_2$ levels might also have to be reduced. Installation of filters to remove impurities from the atmosphere were thought to have contributed to the success of growing *A. thaliana* through an entire life cycle on Salyut-7 (Merkys and Laurinavichyus 1983).

Considering the vigorous growth of our plants, we have every reason to expect that – in spite of the known sensitivity of plants to gravity – wheat and other plants can produce near-normal seed yields in microgravity when all other environmental factors are maintained at appropriate levels (Levinskikh et al. 1999 a, b, 2000). As noted in the Introduction, these expectations were at least partially confirmed by the few seeds produced with Apogee wheat grown in Mir, even with its atmospheric ethylene (Levinskikh et al. 1999 a).

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