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PHYSIOLOGICAL AND GENETIC CHARACTERIZATION OF PLANT GROWTH AND GRAVITROPISM IN LED LIGHT SOURCES

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ABSTRACT

Among the many problems of growing plants in completely controlled environments, such as those anticipated for the space station and the CELSS program, is the need to provide light that is both adequate for photosynthesis and of proper quality for normal growth and development. NASA scientists and engineers have recently become interested in the possibility of utilizing densely packed, solid state, light emitting diodes (LEDs) as a source for this light. Unlike more conventional incandescent or electrical discharge lamps, these sources are highly monochromatic and lack energy in spectral regions thought to be important for normal plant development. In addition, a recent observation by NASA scientists has suggested that infra-red LEDs, that are routinely used as photographic safelights for plants grown in darkness, may interact with the ability of plants to detect gravity.

In order to establish how plants respond to light from these LED light sources we carried out a series of experiments with known pigment mutants of the model mustard plant, *Arabidopsis thaliana*, growing in either a gravity field or on a clinostat to simulate a micro-gravity environment. Results indicate that only red light from the 665 nm LEDs disrupts the ability of normal wildtype seedlings to detect a gravity stimulus. There was no consistent effect found for the far-red (735 nm) LEDs or either of the infra-red (880 nm or 935 nm) LED sources but both showed some effect in one or more of the genotypes tested. Of the five members of the phytochrome multigene family in *Arabidopsis*, only the phytochrome B pigment mutant (hy3) lacked the ability to detect gravity under all conditions. There was no effect of either micro-gravity (clinostat) or the infra-red LEDs on the light induced inhibition of hypocotyl elongation. Measurements of the pigment phytochrome in oats also showed no photoconversion by 15 min irradiations with the infra-red LEDs. We conclude that phytochrome B is required for the perception of gravity and that only red light is able to disrupt this perception. The infra-red LEDs also do not appear to interact with gravity perception in *Arabidopsis*, but caution should be exercised if infra-red LEDs are to be used as photographic safelights for these types of experiments.

SUMMARY

In order to investigate the interaction of light and gravity using LED light sources, we chose several single gene point mutations in *Arabidopsis thaliana* that represented either deletions of individual members of the phytochrome pigment multigene family or loss of phytochrome function. The genotypes used were: (1) hy 8-2, which lacks a light-labile phytochrome A gene product (PhyA) that predominates in dark grown, etiolated tissue and (2) its wildtype ecotype RLD; (3) hy 3, which lacks a light-stable phytochrome B gene product (PhyB) that predominates in light grown, deetiolated tissue and (4) its wildtype ecotype Landsberg erecta (Ler); (5) elf 3, which is an unknown mutation thought to affect phytochrome B function and (6) its wildtype ecotype Columbia (Col).

Approximately 50 seeds of each of these genotypes were surface sterilized and sown on agar in small (3 cm dia.) petri dishes. Velcro™ was attached to the bottoms of the dishes and they were stuck to vertically oriented platters that could be rotated at 1.0 RPM to simulate micro-gravity (clinostat). The clinostats (whether rotated or not) were located inside light-tight, ventillated boxes which were allowed to remain dark for 48 hrs to provide uniform germination. Following germination, the LEDs in each box were turned on to a preset intensity of 100 $\mu mol\ m^{-2}\ s^{-1}$ for the 665 and 735 nm LEDs, 90 $\mu mol\ m^{-2}\ s^{-1}$ for the 880 nm and 65 $\mu mol\ m^{-2}\ s^{-1}$ for the 935 nm LEDs. One box was left in the dark as a control. The seedlings were allowed to grow for 48 hrs, harvested, photographed with a CCD camera and stored as a digital image for hypocotyl length and growth angle (deviation from vertical) analysis.

Results show that there is no interaction between gravity (± clinostat) and the effect of light on the inhibition of hypocotyl elongation in any of the genotypes tested. There is also no inhibition of hypocotyl elongation by the infra-red LEDs. Continuous far-red light strongly inhibits elongation in all genotypes except hy 8-2 whereas continuous red light significantly inhibits all genotypes, including hy 3 and elf 3. The ability to detect gravity (small deviation from the vertical) is normal in the dark and far-red light for all genotypes except hy 3, which behaves agravitropically in all conditions. None of the genotypes are able to detect gravity (large deviation from the vertical) in continuous red light. The data support the conclusion that the Pr form of phytochrome B (which seems to have activity only for this response) is essential for gravity perception. In addition to hy 3, the ecotypes Landsberg erecta and RLD, both of which are normal wildtypes, appear to respond to the 880 nm LEDs while elf 3 appears to respond to the 935 nm LEDs. The latter two responses are not understood, but caution should be used in applications requiring use of infra-red LEDs in gravity experiments.

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I. INTRODUCTION

In order for plants to acclimate to changes in their environment, they must be able to detect such changes and respond by altering internal biochemical and molecular pathways that lead to physiological and morphological differences. One of the most important of these environmental signals is light. Changes in the quality of light provide a plant with information about seasonal variation, spatial orientation, temporal organization, proximity of competing vegetation, etc.. Changes in these parameters are collectively referred to as photomorphogenesis. Photomorphogenetic responses are dependent on the ability of the plant to detect changes in light quality by absorption of light in specific wavelength ranges of the electromagnetic spectrum. Only three pigments have been identified that lead to photomorphogenetic changes: (1) a red/far-red absorbing pigment called phytochrome, (2) a blue light absorbing pigment(s) referred to as cryptochrome(s) and (3) a UV-B absorbing pigment. Of these, only phytochrome has been isolated and characterized.

Phytochrome is present as a multigene family (1) consisting of five separate gene products in *Arabidopsis*. Mutations in two of these genes has indicated that each phytochrome species may control separate photomorphogenetic responses (2). In order to dissect which phytochrome species is responsible for which photomorphogenetic response, we have compared a number of different physiological responses in six different *Arabidopsis* genotypes. One of these genotypes (hy 8-2) (3) lacks the gene product for the light-labile phytochrome A protein, another (hy 3) lacks the gene product of the light-stable phytochrome B protein (4), while a third (elf 3) is a mutant at an unknown locus that has an early flowering phenotype (5). The other three genotypes are all normal wildtype *Arabidopsis* plants with different genetic backgrounds.

Phytochrome is known to regulate the rate of elongation of the embryonic shoot axis known as the hypocotyl. When grown in continuous darkness, the hypocotyls elongate very rapidly; becoming extremely long and spindly. Light inhibits this elongation; producing normal, very short, rosette plants. Continuous, monochromatic far-red light, despite being very dim (<20 µmol m-2 s-1) causes the same level of inhibition as bright white fluorescent light (>200 µmol m-2 s-1). Null mutants lacking the light-labile product of the phytochrome A gene (*phyA*), which predominates in etiolated tissue, completely lack this far-red response and are indistinguishable from the dark controls (3). However, they remain strongly inhibited in both continuous red and white light. Null mutants lacking the light-stable product of the phytochrome B gene (*phyB*), which predominates in light-grown, de-etiolated tissue (4,6), fail to respond to continuous red light

(7). We have noticed; however, that this inhibition is incomplete (unpublished results) either because some other phytochrome species (*phyC*, *phyD* or *phyE*) participates in this response or that our red light source (red phosphor fluorescent filtered through red cellophane) contains enough far-red light to produce *phyA* mediated inhibition. The red LEDs, which do not have any far-red emission, will allow us to distinguish between these alternatives. The *phyB* mutant, like the *phyA* mutant, is also strongly inhibited by white light. We believe that this is a blue light rather than a phytochrome mediated response.

A recent report (8) demonstrated that the shoots of wildtype *Arabidopsis* seedlings were able to detect, and respond to, gravity when grown in complete darkness , but were unable to do so when irradiated with continuous red light. Interestingly, the *phyB* mutant hy-3 was unable to detect gravity even in continuous darkness. The red light induced disruption of the gravitropic response was found to be red/far-red reversible, confirming that phytochrome was required. Since the mutant only lacks phytochrome B, and since it was unable to detect gravity in the dark (where only the "inactive" Pr form of phytochrome is present), it was argued that the Pr form of phytochrome B was required for gravitropic perception in Arabidopsis shoots. Red light would lead to a reduction in the pool of "active" Pfr in the wildtype and increase the pool of "inactive" Pr; causing it to lose its ability to respond to a gravity stimulus. This is an extremely controversial conclusion because it is the only instance where the Pr form of the phytochrome molecule has been shown to have biological activity. They further confirmed this by showing that hy-2, which is defective in chromophore biosynthesis leading to a reduction in all phytochrome species, also failed to detect gravity in the dark, but could be rescued by feeding the immediate precursor of the chromophore, biliverdin.

Scientists at KSC and the Bionetics Corporation have discovered that energy from infra-red emitting LEDs is able to cause a number of responses in oat seedlings that may be attributible to phytochrome (9, Appendix). They found that, when seedlings were irradiated with either 880 nm or 935 nm LEDs, mesocotyl (embryonic shoot in grasses equivalent to the hypocotyl in *Arabidopsis*) growth was inhibited, coleoptile (embryonic sheath covering the leaves) growth was increased and leaves emerged earlier than seedlings grown in darkness. In addition, the IR irradiated plants had mesocotyls oriented in an orthogravitropic (growth parallel to, but away from the gravity vector) direction while the dark controls were oriented in a diagravitropic (perpendicular to the gravity vector) direction. This has caused concern since the use of such LEDs as "safelight" sources for infra-red sensitive cameras has been recommended for monitoring biological experiments aboard the Space Station (10).

We have; therefore, undertaken an investigation of the phytochrome involvement in both the growth responses and the gravitropic responses in *Arabidopsis* using red (665 nm), far-red (735 nm) and infra-red LEDs (both 880 nm and 935 nm). Also, in order to characterize which members of the phytochrome multigene family are responsible for these responses, we have examined all of the responses in the phytochrome pigment mutants hy 8-2, which lacks phy A, hy-3, which lacks phyB and elf-3, which is unable to respond to phyB stimulation.

II. EXPERIMENTAL PROCEDURES

2.1 SEEDS

Six different genotypes were used during the course of these experiments. The elf-3 seeds were obtained from Dr. D. Rye Meeks-Wagner at the University of Oregon, hy 8-2 seeds were obtained from Dr. Peter H. Quail at the University of California at Berkeley and the hy-3 seeds were obtained from Richard Amasino at the University of Wisconsin. All seed stocks were maintained by selfing the homozygous recessive plants in the growth chamber facility at the University of Maryland. All seed were stored in a dry condition at 4 °C until use.

2.2 SURFACE STERILIZATION

Approximately 300 seed of each genotype were added to 15 ml sterile, disposable Falcon tubes to which was added 10 ml of a 20% Chlorox™ solution (1.5% $NaHClO_4$). These were stirred on a vortex stirrer and allowed to incubate for 20 min. at room temperature in a laminar flow hood. At the end of this period all of the seed was rapidly transferred by filtration through a presterilized Whatman™ No. 1 filter paper and washed three times with 10 ml each of sterile distilled water. The filters were then removed and allowed to air dry in the laminar flow hood overnight. This procedure was found to be necessary to stimulate germination of seeds in darkness which otherwise have a light requirement.

2.3 EXPERIMENTAL PROTOCOL

2.3.1 STATIONARY. Approximately 50 seed of each genotype were transferred to small (3.5 cm) sterile, disposable petri dishes containing 0.7% (w/v) Sigma No. A-7002 agar in deionized water under a dim (<2.0 µmol m^{-2} s^{-1}) green (2 x 15 in green phosphor fluorescent tubes wrapped with several layers of green cellophane) safelight in a photographic darkroom. The dishes were then sealed with Parafilm™ and a strip of Velcro™ was applied to the bottom of each dish. The dishes were transferred in darkness to one of five specially constructed 245 cm x 368 cm light-tight wooden boxes that contained an LED board at one end and a 15 cm diameter round Velcro™ covered platter at the other. The LED end of the chamber was separated from the remainder of the chamber by a 1/8 in thick clear acrylic barrier and air was circulated over the LEDs using a centrifugal blower with a light trapped opening. The platter was mounted vertically and attached to an electrical motor mounted outside of the box. The boxes were maintained at 23 °C inside a Conviron E-36 growth chamber (Dark, 880 and 935 nm LEDs) or in the photographic darkroom (red and far-red LEDs).

Seeds were allowed to germinate in darkness for 48 hrs before the

lights were turned on so that the light conditions would not influence the time or percentage of germination. Following germination the LEDs were switched on to preset irradiances corresponding to calibrated currents on the power supplies. The plants were then irradiated for an additional 48 hrs and then removed in darkness, covered with aluminum foil and placed at 4 °C. As soon as possible thereafter, each dish was captured as a digital image using a CCD camera and the image stored in the computer. The dished were then returned to 4 °C in the dark.

2.3.2 CLINOROTATED. One half of the experimental series was treated in exactly the same way as those described above for the stationary response, but after placing the dishes on the platter, the electrical motor connected to it was switched on. The motor was chosen to provide a constant 1.0 RPM throughout the experiment. Since seedlings germinated on agar in a vertical direction grow along the surface of the agar, clinorotated seedlings experience a uniform gravitational field in the direction of growth.

2.4 LIGHT SOURCES

The 880 nm LEDs consisted of 100 Honeywell # 840-3470-001 gallium-aluminum arsenide diodes (Micro Switch Division, Richardson, TX) mounted in a 21 cm^2 matrix and provided a maximal fluence rate of 90 $\mu mol\ m^{-2}\ s^{-1}$. The 935 nm LEDs consisted of the same configuration as the 880 nm LEDs but were gallium arsenide (Honeywell # 840-3445-004) and provided a maximal fluence rate of 65 $\mu mol\ m^{-2}\ s^{-1}$. Red LED light was obtained from a monolithic array of diodes sold commercially by Quantum Devices, Inc. (Barneveld, WI) as their Model QBE AM 2000 which provided 100 $\mu mol\ m^{-2}\ s^{-1}$ at 8.0 Amps measured at the center of the clinostat. The far-red LEDs were obtained as part of a prototype array containing both red and far-red LEDs from NASA Ames, but were also fabricated by Quantum Devices, Inc. 144 of the far-red LEDs were removed and mounted in a single circuit board and set at 2.3 Amps to provide 100 $\mu mol\ m^{-2}\ s^{-1}$ at the center of the clinostat. The spectral distribution, measured with an LI-1800 spectroradiometer (LiCor, Lincoln NE), of all of these sources is shown in Fig. 1.

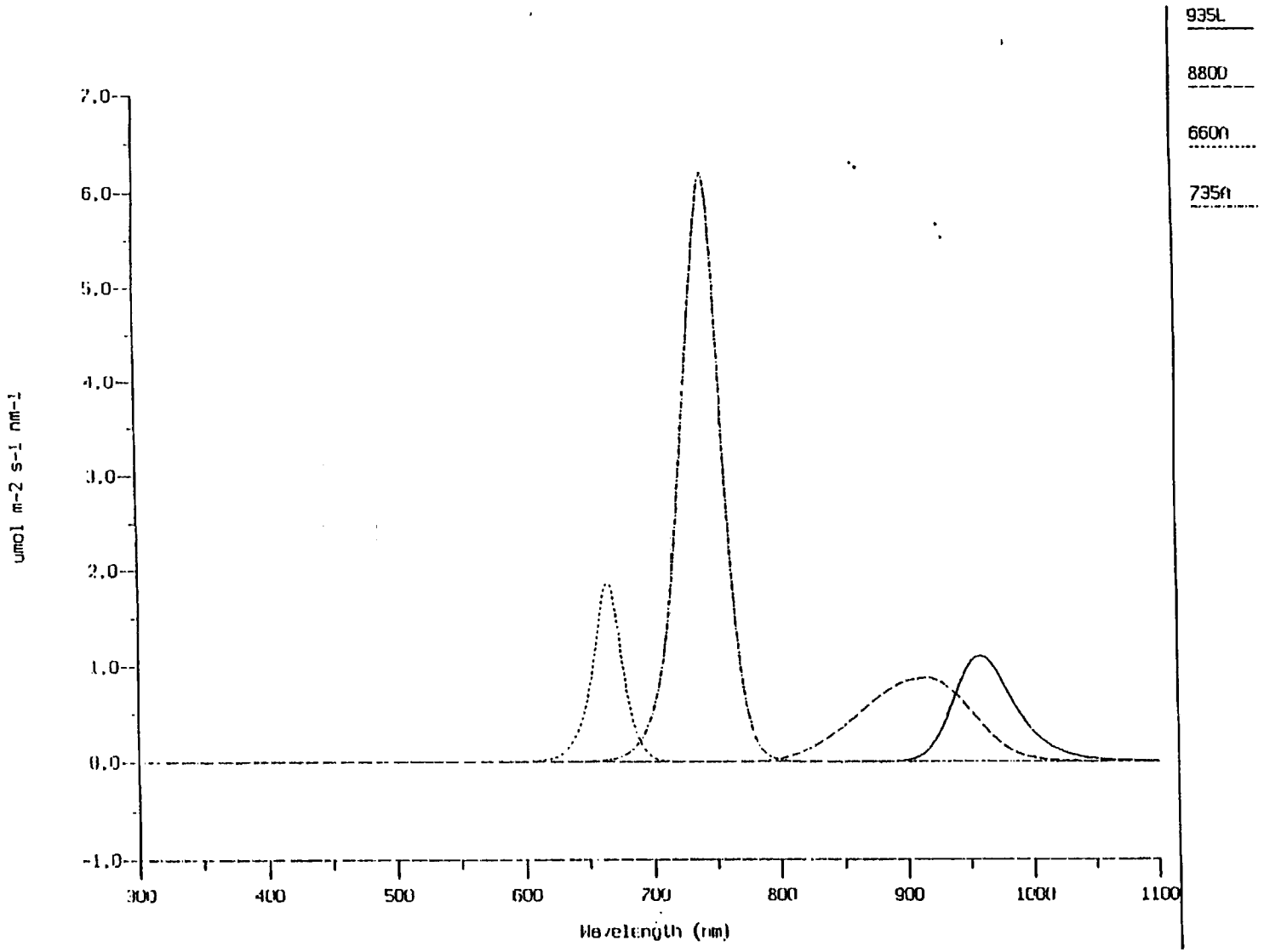


Figure 1

Emission Spectrum of the LED Light Sources

2.5 IMAGE ANALYSIS

Immediately following harvest, the petri dishes were opened and the Velcro removed from the bottom to allow the seedling images to be captured by CCD camera and the digitized image was stored on a Macintosh Quadra 950 computer. A millimeter ruler image was captured next to each dish in order to calibrate the hypocotyl length measurements. Each image was then analyzed for hypocotyl length and angle using NIH Image software, a public domain program developed for Macintosh by Wayne Rasband of the National Institutes of Health. Each seedling is simultaneously analyzed for length and angle and the results transferred to Excel for statistical analysis. All experiments were repeated three times and the means and standard deviations calculated.

2.6 PHYTOCHROME MEASUREMENTS

Phytochrome was measured using a custom-built ratio-spectrophotometer as the difference in absorbance between 660 nm and 730 nm before and after actinic irradiation with red and far-red light. Oat seedlings were grown on moistened Kimpack™ in the dark for 4-6 days. At the end of this time, the upper 1.5 cm of several hundred coleoptiles were harvested and 1.0 g of fresh weight were placed in petri dishes on ice. These samples were then transferred to each of the LED light conditions and irradiated for 15 min. on ice to prevent thermal destruction of the labile Pfr pool. Following irradiation the coleoptiles were chopped into 0.5 mm sections with a razor blade and placed into a cylindrical aluminum cuvette which is open at the top and has a clear plastic window on the bottom. This cuvette was the placed in the ratio-spectrophotometer and the difference in absorbance recorded. The sample is then irradiated from above by a high irradiance far-red source for 1.5 min. and then the difference in absorbance was immediately measured again. This was then repeated for five cycles alternating red and far-red irradiations between measurements. The Pfr/Ptotal photoequilibrium was then calculated.

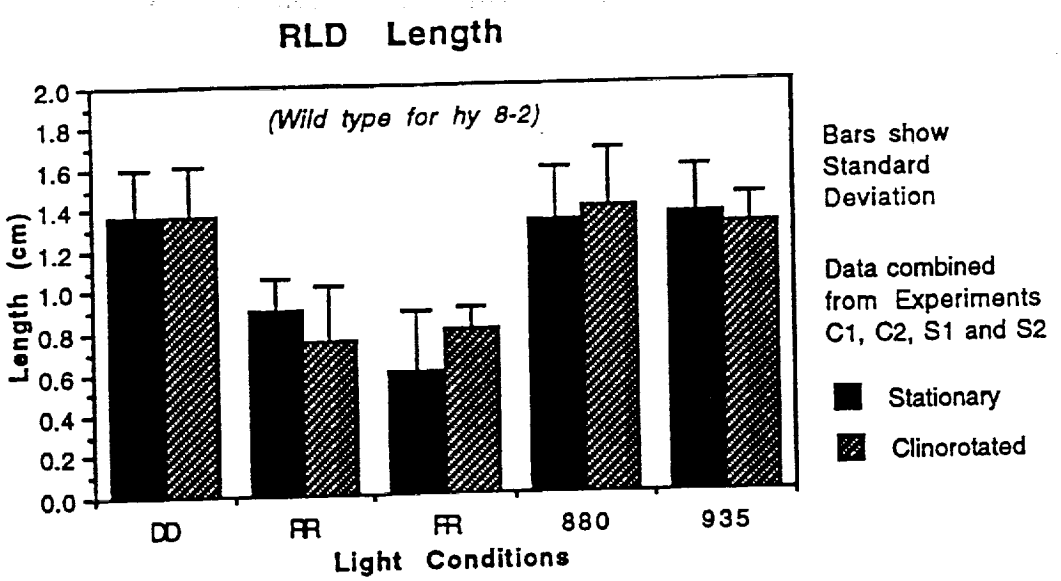
III RESULTS AND DISCUSSION

3.1 HYPOCOTYL ELONGATION

When allowed to germinate and grow in continuous darkness, virtually all plants will respond by elongating very rapidly and by displaying a whole host of characteristics that are typical of growth in the absence of light. This condition is known as etiolation and is a transient condition since, without light, plant cells elongate but do not divide to produce new cells for growth. Thus they can survive only until internal energy stores are exhausted. Light immediately inhibits this rapid cell elongation, initiates the synthesis of chlorophyll and causes leaves to expand and begin photosynthesizing to provide new energy from external sources. While these changes involve the synthesis of chlorophyll and the development of the photosynthetic apparatus, the light signal is not detected by chlorophyll itself. The pigment responsible is phytochrome, a photointerconvertible photoreceptor molecule that exists in two isomeric forms. One of these forms, which has an absorption maximum in the far-red region of the spectrum at 730 nm, is known as Pfr and is thought to be the active form of the molecule. The other, which has an absorption maximum at 667 nm is known as Pr and is considered to be inactive. Absorption of light by one form converts it to the other form and so a photoequilibrium is established by irradiation with any light source. The relative proportion of Pfr to the total pool of phytochrome is important in determining the extent of the response to light.

3.1.1 HY 8-2. Not only does phytochrome exist in two isomeric forms, but there are at least five different phytochrome genes expressed in *Arabidopsis*. One of these predominates in etiolated plants and is known to be encoded by the phytochrome A gene (11) and is characterized by being light labile as Pfr and so it disappears very rapidly following irradiation. It is also known to be both necessary and sufficient for the far-red mediated inhibition of hypocotyl elongation. This was deduced following the isolation and characterization of a mutant called hy 8, especially the hy 8-2 allele, which was found to have a stop codon in the gene encoding phytochrome A (3) resulting in the complete loss of inhibition by far-red light.

Results reported in Fig. 2 show that hy 8-2 elongates normally in the dark (DD) as well as after 48 hrs of irradiation with far-red (FR) light. In contrast, the wildtype RLD, with which it is isogenic, is completely inhibited by 48 hrs of far-red light. There is also no apparent inhibition by either of the IR LEDs (880 or 935 nm) in either the mutant or the wildtype, but both are strongly inhibited by 48 hrs of irradiation with red (RR) light. There is also absolutely no effect of clinorotation on any of



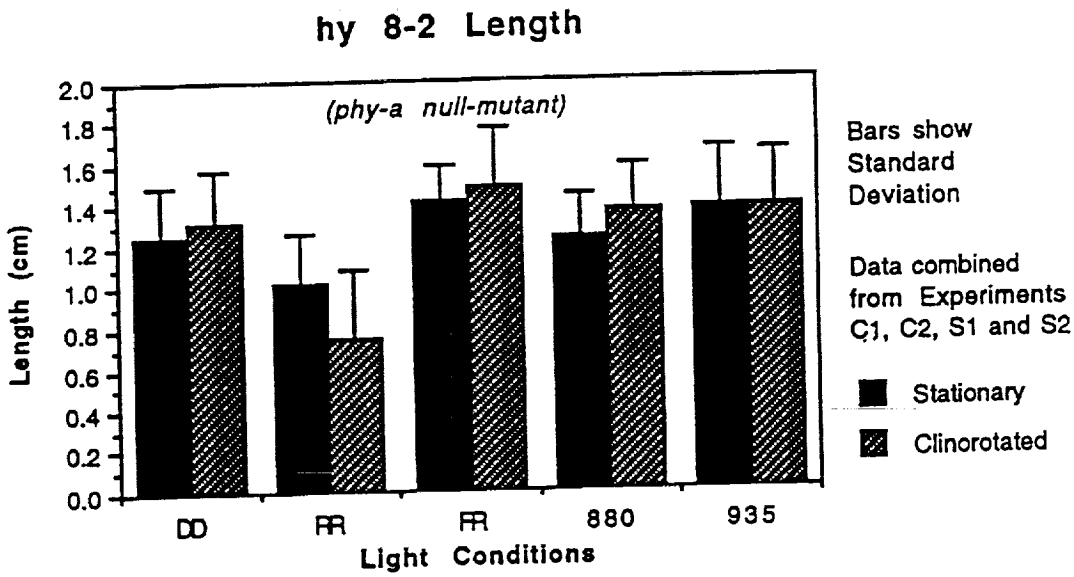


Figure 2

Hypocotyl Elongation of RLD and hy 8-2 in various light conditions with and without clinorotation

these responses. Thus phytochrome A is responsible for the detection of continuous far-red light but not continuous red light and neither infra-red nor gravity interact with this response.

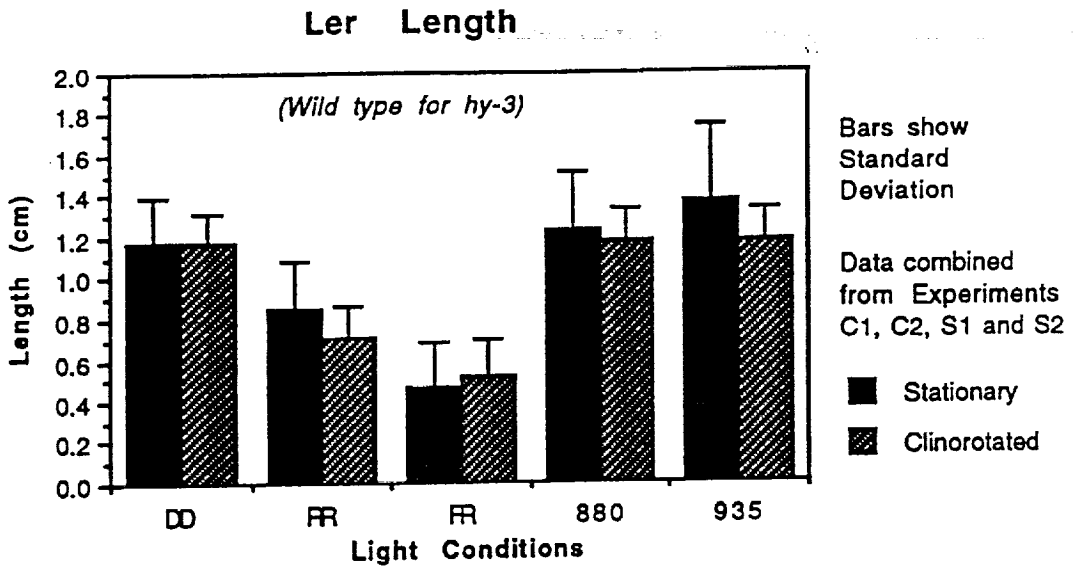
3.1.2 HY-3. Hy-3 was originally isolated in 1980 by Maarten Koornneef (12) as one of a group of mutants that developed long hypocotyls in the light that were characteristic of etiolated plants. It was subsequently found (6) that this mutant was devoid of phytochrome B and was characterized by its failure to be inhibited by continuous red-light (13). Fig 3 shows that the response of hy-3 to red light (RR) is completely normal. This suggests that at high irradiances (100 μmol m^{-2} s^{-1}) that some other type of phytochrome can assume this function, possibly even phytochrome A as suggested recently by Reed et al. (14). Again, as with hy 8-2, there was no significant effect of either the infra-red LEDs nor gravity for both the mutant and its isogenic wildtype, Landsberg erecta.

3.1.3 ELF-3. When this mutant was isolated in 1992 (5), it was characterized as an early flowering mutant, but it was noted that it also produced very long hypocotyls when grown under short photoperiods. In this respect, these plants resembled the hy mutants, especially hy-3. However, when the levels of phytochrome were examined on Western blots, they were found to have normal levels of phytochromes A, B and C (Zagotta, Meeks-Wagner and Quail, unpublished results). In addition, the elf-3 mutation was found not to map to any of the known chromosomal locations for phytochrome (5). When compared under our conditions, elf-3 was found to behave exactly like hy-3 (Fig. 4) and did not differ significantly from its isogenic wildtype Columbia. Also, as was found for hy 8-2 and hy-3, the was no effect detected for either gravity or infra-red irradiation. Since both elf-3 and hy-3 appear to be phytochrome B mutations, we have suggested that elf-3 is a mutation in one of the biochemical events downstream from photoreception but within a common pathway leading to a number of phytochrome B mediated responses. The normal inhibition of hypocotyl elongation in elf-3 is also interpreted as being due to overlapping functions of photoreceptors, especially at high irradiances.

3.2 GRAVITY PERCEPTION

Although it is questionable to what extent rotation on a clinostat simulates the effects of micro-gravity, it at least provides a first approximation where the effects of a gravitational field are at least equalized in all directions. Deviations from a uniform direction of growth may reasonable be assumed to conform to a failure to perceive a gravity stimulus.

3.2.1 HY 8-2. When hy 8-2 was grown on the clinostat it grew randomly in all directions (Fig. 5) in all of the light treatments. This was also true for its wildtype RLD and so may be regarded as an internal control for the interaction of light with



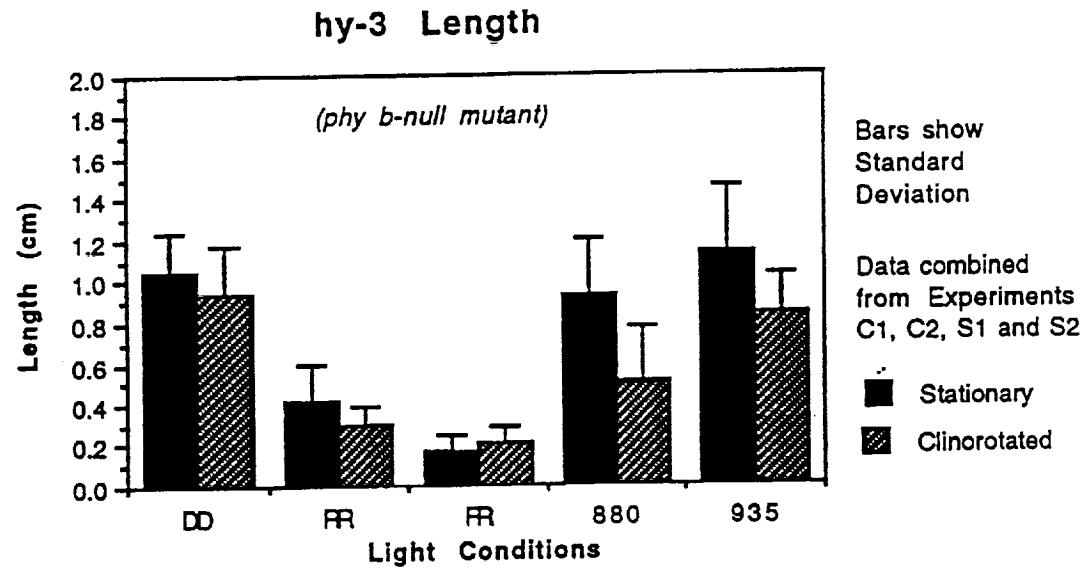
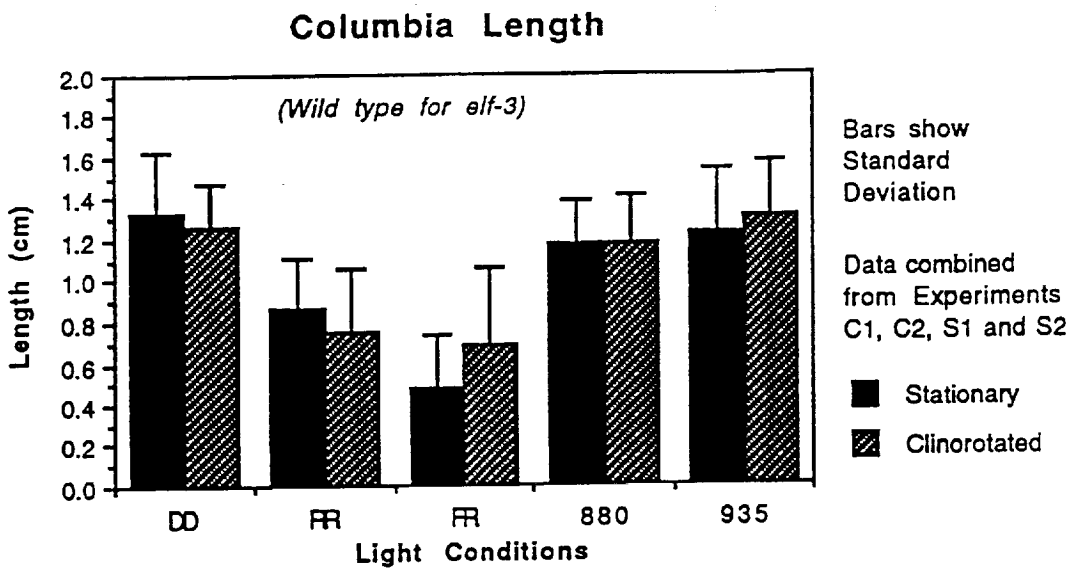


Figure 3

Hypocotyl Elongation of Ler and hy-3 in various light conditions with and without clinorotation



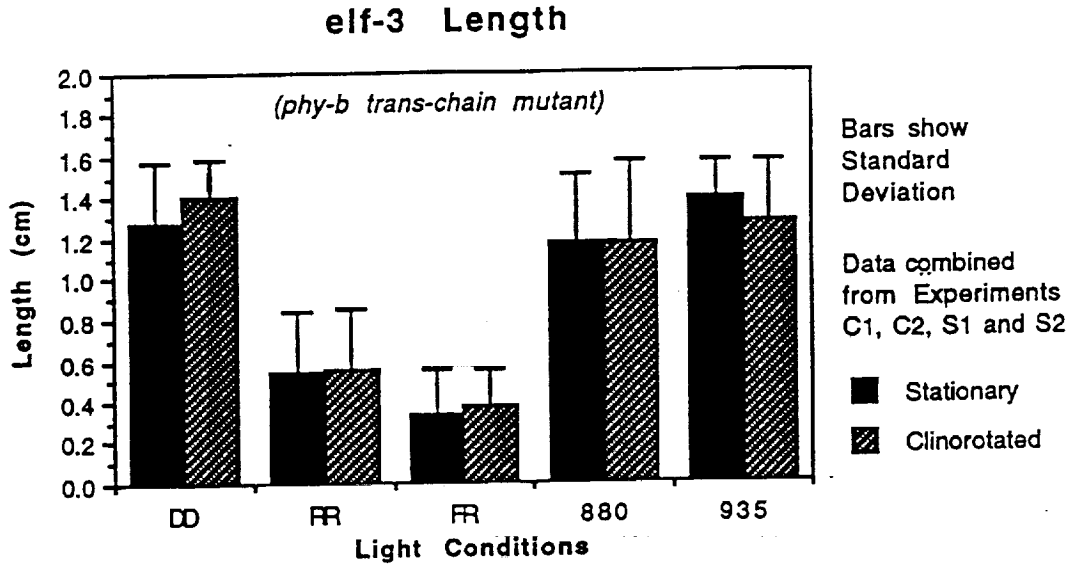
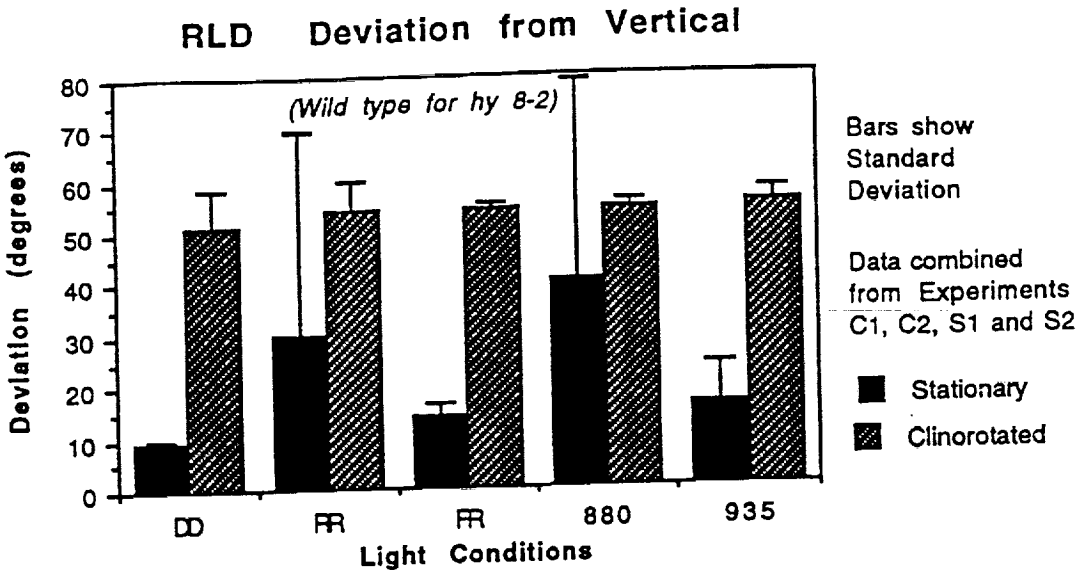


Figure 4

Hypocotyl Elongation of Col and elf-3 in various light conditions with and without clinorotation



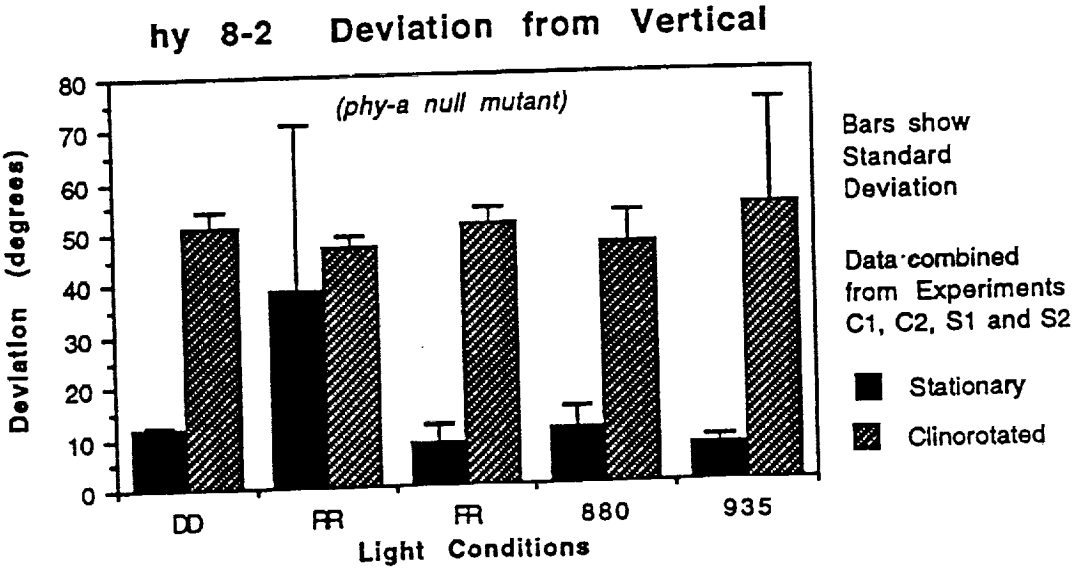


Figure 5

Growth angle of RLD and hy 8-2 in various light conditions with and without clinorotation

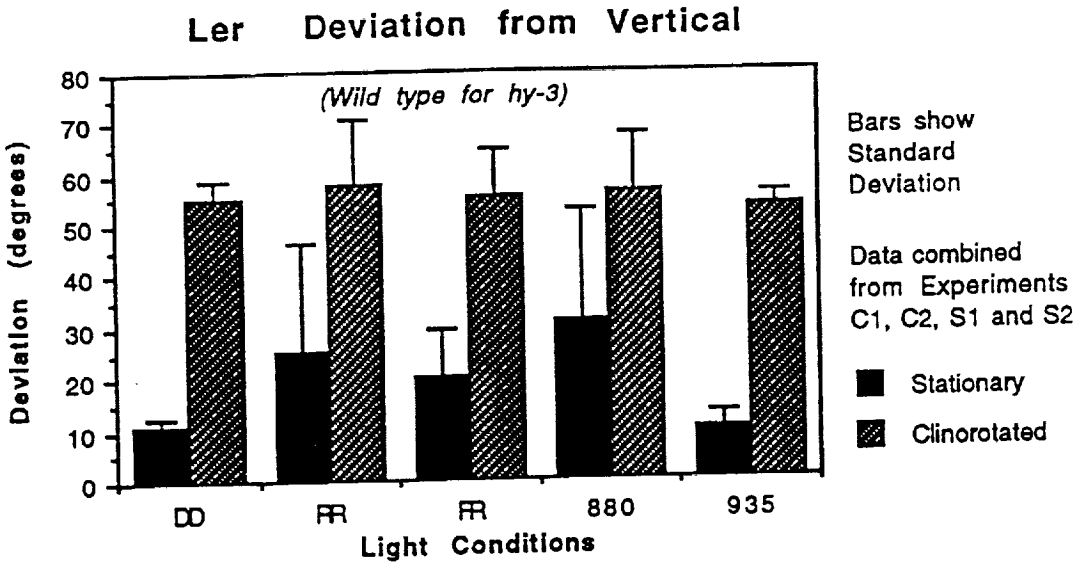
gravity perception. When the clinostat was switched off and the seedlings grew in a stationary vertical orientation, both hy 8-2 and RLD were able to orient to the gravity vector in continuous darkness (DD), far-red (FR), 880 nm (880) and 935 nm (935) LEDs. They were both; however, completely randomized when grown in continuous red (RR) light. Thus, the ability to respond to gravity is not a phytochrome A mediated response because both the mutant and wildtype are normally oriented in continuous far-red light which was clearly demonstrated to differ in section 3.2.1. Thus, the disruption of gravity perception by red light must be mediated by another species of phytochrome. The failure of either 880 nm or 935 nm LED energy to disrupt gravitropism clearly indicates that they do not interfere with gravity perception through phytochrome A.

3.2.2 HY-3. Unlike hy 8-2, hy-3 is completely agravitropic under all conditions including darkness (DD) (Fig. 6). Landsberg erecta, on the other hand only fails to respond to gravity only when grown in continuous red (RR) and so behaves like hy 8-2 and RLD. This confirms the report of Liscum and Hangarter (8) that phytochrome B is required for the perception of gravity. It is also consistent with the suggestion that the Pr form of phytochrome B is required for this response. Here, the infra-red LEDs, while they both fail to respond to gravity, nevertheless behave exactly like the dark control. Thus, there is no apparent effect of infra-red on the gravity response in hy-3 either.

3.2.3 ELF-3. If elf-3 is a mutation in the biochemical transduction chain initiated by phytochrome B, as is apparent in section 3.1.3, then it should behave exactly like hy-3. Figure 7; however, shows a very different picture. Elf-3 is completely normal when grown in continuous darkness (DD) but is randomized when grown in continuous red (RR) light. The responses to far-red (FR) and infra-red (880 and 935) are; however, ambiguous. They appear to be partially gravitropic under these conditions, but are clearly not as agravitropic as hy-3. The ability to perceive gravity normally in the dark in elf-3 suggests that, while phytochrome B may be required for this response, the light dependent transduction chain is not. Hence the interpretation by Liscum and Hangarter (8) that the Pr form of phytochrome B is required is not supported by these data. The effect of red light on the disruption of this response remains to be explained, but it does not appear to be a simple case of converting the Pfr form of phytochrome B back to the Pr form.

3.3 PHYTOCHROME MEASUREMENTS

Although only elf-3 showed a partial effect when plants were grown in infra-red energy, the question of whether the 880 nm and 935 nm LEDs could photoconvert phytochrome from Pr to Pfr was addressed by measuring the photoequilibrium directly. Unfortunately, the



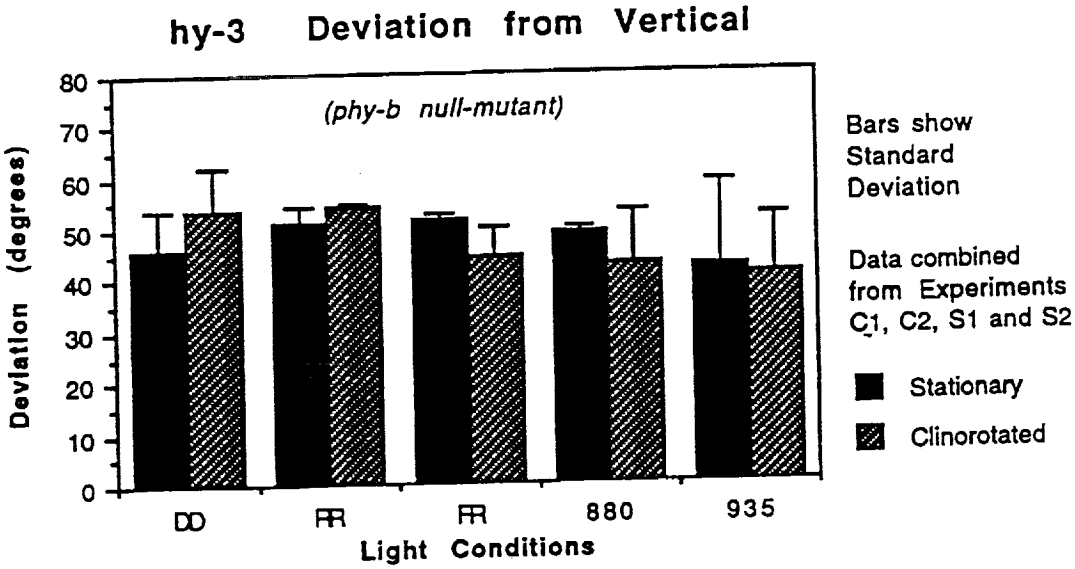
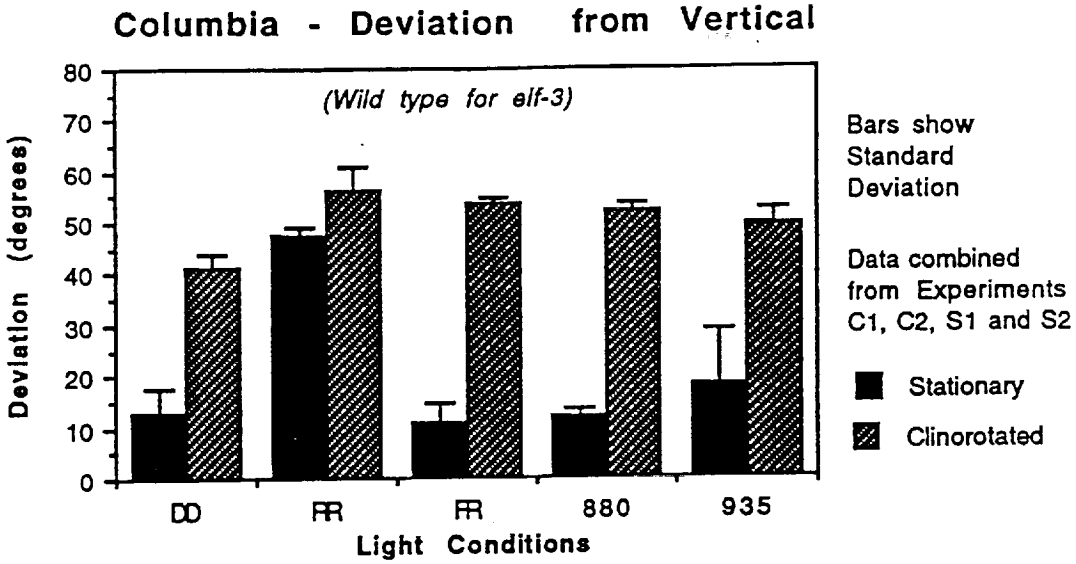


Figure 6

Growth angle of Ler and hy-3 in various light conditions with and without clinorotation



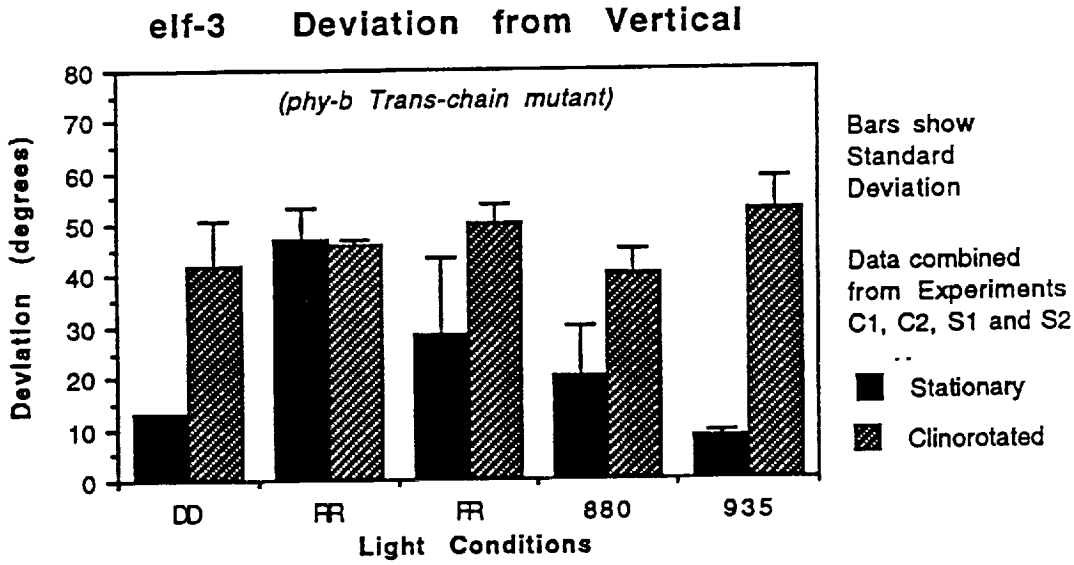


Figure 7

Growth angle of Col and elf-3 in various light conditions with and without clinorotation

concentrations of phytochrome in *Arabidopsis* are too low for accurate measurement of phytochrome *in vivo* and so we were forced to make these measurements in oat seedlings. Table 1 shows the results of 15 min. irradiations of oat coleoptiles with each of the LED light sources. Since phytochrome is synthesized as Pr in the dark, there is no Pfr evident in dark-grown, etiolated plants (6d DD) or in plants that had been sham irradiated in a box with the LEDs turned off (6d DD + 15 min. DD LED). The far-red LEDs produce about 7.5 % Pfr because of the overlap in the absorption spectra of Pr and Pfr in the far-red (6d DD + 15 min. FR LED). The red LEDs (6d DD + 15 min. RR LED) produced 86 % Pfr, which is the maximal theoretical photoequilibrium obtainable due to the spectral overlap. Neither of the infra-red LEDs produced any measurable Pfr following 15 min. of irradiation. It is possible that some Pfr would have been formed with longer irradiations, but this is technically difficult to test because of the need to irradiate on ice. An attempt will be made to first irradiate the coleoptiles with red light followed by infra-red light to see if the photoequilibrium can be lowered.

TABLE 1

Phytochrome Measurements of 1.0 g Fresh Weight Oat Coleoptiles Following Irradiation with 15 min. of Light from Various LED Sources

TREATMENT 6d DD + 15 min.	TOTAL PHYTOCHROME ($\Delta\Delta A \times 10^{-3}$)	Pfr (% of Ptot)
DD	49.53	0.0
Far-Red LEDs	51.66	7.4
Red LEDs	60.59	86.0
880 nm LEDs	58.43	0.0
935 nm LEDs	60.21	0.0

IV SUMMARY COMMENTS

Although the results presented in this report are to some extent disappointing in the sense that they failed to confirm an interaction between the infra-red LEDs and gravity perception, they provide a much clearer definition of the observations made in oat seedlings. The two possible explanations for those observations were that (1) the infra-red LEDs produced contaminating radiation of shorter wavelength and (2) that phytochrome could be excited by the infra-red wavelengths emitted by the infra-red LEDs. Both of these possibilities can be ruled out in *Arabidopsis* since the infra-red LEDs had no effect on either growth or gravity perception. This does not rule out the possibility that oat is more sensitive to infra-red radiation and shows a response that cannot be detected by *Arabidopsis* or that the phytochrome system in oats is different than that in *Arabidopsis*. It does suggest; however, that the observed effects in oat seedlings may not be mediated by phytochrome but by some pigment that is present in oat but not in *Arabidopsis*.

Finally, the most unexpected result found during this study was that hypocotyl elongation in hy-3, which lacks phytochrome B, nevertheless was inhibited by red light. We believe that this is due to the very high irradiances that were achieved by the red LEDs which were 5-7 times higher than those used earlier. Apparently at such high irradiances another species of phytochrome is able to assume the function normally mediated by phytochrome B. This may be phytochrome A, even though hy 8-2 is also inhibited by red light, because hy 8-2 has normal levels of phytochrome B. It may; however, be mediated by one of the other species of phytochrome (phy C, D or E) that have no known function. We will perform fluence response tests with red light to establish whether hy-3 simply has an altered sensitivity. Understanding this response is important since red light irradiances that will be used for life cycle experiments for the Space Station will be well above the 100 μmol m^{-2} s^{-1} used in this study.

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VI. APPENDIX

Running Head: PRELIMINARY DRAFT

INFRA-RED RADIATION EFFECTS ON OAT SEEDLINGS

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PRELIMINARY DRAFT

Infra-red Light-emitting Diode Radiation Causes Gravitropic and Morphological Effects on Dark-Grown Oat Seedlings

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2 Abbreviations: IR, infra-red; LED, light-emitting diode; nm, nanometer (wavelength); cv., cultivar;

ABSTRACT

Oat (*Avena sativa* cv Seger) seedlings were irradiated with two sources of infra-red (IR) light-emitting diode (LED) radiation (peak wavelengths 880 and 935 nm) passed through a visible-light blocking filter (blocks wavelengths below 800 nm). IR LED irradiated seedlings exhibited differences in growth (i.e., reduction in mesocotyl tissue length, increase in coleoptile length and advanced leaf emergence) when compared to seedlings grown in darkness at the same temperature. Further, IR LED irradiated seedlings exhibited an orthogravitropic response in the mesocotyl while dark-grown seedlings exhibit a diagravitropic response. The oat seedlings in this study perceived IR LED radiation. These findings stress the importance of careful verification testing before the use of IR LEDs as a safe-light for photosensitive plant response experiments.

INTRODUCTION

Infra-red (IR) light-emitting diodes (LEDs) have been used in several plant experiments as a "safe-light" source for IR sensitive cameras (1-3, 5, 7, 13). Dark photography was preferred in these experiments on circumnutation and gravitropism because it allowed plant movements to be observed while eliminating the complicating phototropic responses caused by flash (or light) photography. An IR camera system for dark cycle monitoring of biological specimens also has been recommended for the NASA Space Station (9). The IR LEDs in this viewing system provide illumination to the IR camera in the near infra red (780 - 1000 nm) region of the spectrum. Because of the relatively narrow band-width of the LED radiation, and because the IR LED wavelengths are outside the visible region of the electromagnetic spectrum, it has been assumed their use in dark photography systems would have no effects on plants.

In previous studies, we have tested IR LEDs and found them safe with regard to phototropic influences on dark-grown oat seedlings. However, mesocotyl tissue of oat seedlings grown under long-duration exposure to IR LED radiation (filtered through an IR-transmitting, visible-light-blocking filter) exhibited an apparent orthogravitropic response whereas the dark-grown oat seedlings' mesocotyl exhibited an apparent diagravitropic response.

Two possible explanations for the observations are considered. One possibility addressed here is that both the IR LEDs and the visible-blocking filter may provide extremely minute, but sufficient quantities of red light wavelengths to the plants to account for the observed gravitropic responses. Phytochrome, has been implicated in red light-induced gravitropism (8, 11, 12) and the observed morphological changes in the seedlings (suppressed mesocotyl growth and advanced leaf emergence) are consistent with known phytochrome-induced changes (4, 6). However, the absorption spectra of purified oat phytochrome (P_{fr}) includes a "tail" which extends into the near infrared region (10). Thus, a second possible explanation is that IR light, rather than red, through absorption by P_{fr} is causing the observed response.

In this report, we describe two series of experiments which demonstrate that the radiation from IR LEDs is not imperceptible to dark-grown oat seedlings. Also described is the analysis of the LED spectral quality output. (For clarification, when IR light and IR radiation are discussed here, this only refers to the spectral radiation in the near-infrared portion of the electromagnetic spectrum.)

MATERIALS and METHODS

Plant Material and Growth

Oat (*Avena sativa* cv Seger) seeds were planted in a moistened peat/vermiculite mix (Metro-Mix 220, Grace Sierra Horticultural Products Company, Milapitas, CA) so that the brush of the seed was even with the soil line. Seeds were planted with the seed's point-of-attachment directed downward into the soil, and the seed coat crease oriented 90-degrees from the direction of IR LED irradiation. The germinating oat seedling, when grown in the dark, directs shoot growth away from the seed in the direction of the seed coat crease. This directional growth (possibly a nastic response) was exploited to differentiate between an apparent phototropic response and a morphological growth pattern. By uniformly orienting the seeds, the natural direction of coleoptile growth was 90 degrees from the IR light source. For the first set of experiments, seeds were planted in 147 cm (6 inch) standard plastic pots, covered with aluminum foil to retain soil moisture. Holes were punched through the aluminum foil to provide openings for the seeds. In the second set of experiments, seeds were planted in soil trays made of black anodized aluminum as described by Johnsson *et al* (7). The soil trays were placed inside light-tight plant modules with windows made of IR transmitting, visible-light-blocking acrylic (Rohm and Haas acrylic # 2650 Rohm and Haas, Philadelphia, PA). The plant chambers were maintained at a temperature of 22.5 $\pm$ 1 ^{o}C in all experiments.

Experimental

In the first set of experiments, the IR LED radiation of either 935 nm or 880 nm, (depending on treatment) was passed through a clear acrylic window inside a 245 cm x 368 cm wooden dark box. The wooden dark boxes were placed inside a temperature controlled growth chamber. The seedlings received continuous IR LED treatment from time of planting to age 120 hours. Controls were grown in a dark box identical to the IR LED treatment boxes. The 880 nm LEDs used in the study were made of aluminum gallium arsenide (Honeywell # 840-3470-001, Micro Switch Division, Richardson, TX) and provided a fluence rate of 90 μmol$\cdot$m$^{-2}\cdot$s^{-1}. The 935 nm IR LEDs used were made of gallium arsenide (Honeywell # 840-3445-004, Micro Switch Division, Richardson, TX) and provided a fluence rate of 65 μmol$\cdot$m$^{-2}\cdot$s^{-1}. In both 935 nm and 880 nm LED boxes, one-hundred LEDs were arranged in a 21 cm square matrix. There were 20 seeds planted per pot with one pot per treatment. This experiment was performed 5 times.

In the second set of experiments, to increase our confidence that neither red nor far-red light

were contaminating the experimental set-up (through very minute amounts possibly being emitted by the IR LEDs) the 880 nm LED treatment was eliminated and an IR filter was added to the 935 nm LED system. The IR transmitting visible-light-blocking filter-windows on the plant modules provided a cut-off of light wavelengths below 800 nm. Oat seedlings were grown for 58 hours in darkness, then exposed to 935 nm IR LED radiation passed through the IR filter from age 58 to 84 hours. At the temperature of 22.5 °C, the seedlings are just emerging above the soil line at age 58 hours. This shorter growth time (and shorter length) allowed the seedlings to be grown inside the small (but available) light-tight IR-filter plant modules, as described above. The IR transmitting filters reduced irradiance from the 935nm LEDs to a fluence rate of 15 $\mu mol \cdot m^{-2} s^{-1}$. Dark controls were also grown inside the light-tight modules, wrapped in aluminum foil to block IR radiation from entering through the IR filter windows. There were 9 seeds per plant module, and 2 plant modules per treatment. This experiment was performed 8 times.

Measurements

Plants were removed from the treatment dark boxes and photographed from the side using an IR sensitive CCD camera (Model 4TN2505, General Electric, USA). The image was digitized and stored on computer. Plants were then removed from their containers and length of coleoptile, mesocotyl and leaf tissue were measured. Plant angle measurements were made from the digitized image of the seedlings with the aid of an Apple Macintosh computer image analysis program (NIH Image). In the first set of experiments, tip-to-base angles were measured in the longer, 120 hour old seedlings. In the second set of experiments, the angle of orientation with respect to vertical was measured along the bottom third of the seedling shoots (mesocotyl tissue region) in the shorter, 85 hour old seedlings. Measurements of light quality were made with an LI-1800 spectroradiometer (LiCor, Lincoln, NE). The spectroradiometer scanned between 300 - 1100 nm, in 2 nm intervals, to determine the spectral quality and quantity of the LEDs. The dark control box was also measured to establish a baseline and to confirm that there was no light contamination in the visible or IR range.

RESULTS

Plant growth responses

As expected, growth of the dark-grown seedlings was uniformly directed away from the seed in the direction of the seed coat crease. Also as expected, there was no phototropic bending response of the seedlings was observed in any of the treatments.

In the first set of experiments, growth measurements of the 120 hour-old seedlings showed seedlings grown in the presence of 880 nm IR LEDs were shorter and had a lower percentage of mesocotyl tissue than seedlings grown under 935 nm LEDs or in darkness (Table I). Although total lengths of dark-grown seedlings and 935 nm seedlings were not significantly different, seedlings grown in the presence of 935 nm LEDs had a lower percentage of mesocotyl tissue than the dark-grown controls. The 880 nm IR LED treated seedlings were more advanced in leaf emergence and unfurling than 935 nm IR LED treated seedlings or dark-grown seedlings. Angle measurements show that there was no significant difference in seedling tip-to-base angles between the two IR irradiated treatments. However, both the 935 nm and 880 nm LED treatment seedling angles were significantly different from the dark-grown seedling angles (Figure 1) at the 0.05 confidence level (Table I.)

In the second set of experiments, growth measurements of the 85 hour old seedlings showed oat seedlings grown in the presence of IR radiation (935 nm) were shorter and had significantly less mesocotyl tissue than seedlings grown in darkness (Table I). Angle measurements show that seedlings treated with 935 nm IR LED radiation passed through an IR filter grew straighter than dark-grown seedlings .

Light quality measurements

Spectroradiometer measurements show that no visible light (300 - 700 nm) was present in either IR system or the dark control (Figure 2). Closer examination of the tail regions of the spectral irradiance curves (Figure 3) show that the 880 nm LED source begins radiating quantities of less than 0.1µmol m-2 s-1 over the range of wavelengths below 800 nm and the 935 nm LED source begins radiating quantities of less than 0.1µmol m-2 s-1 below 900 nm. Measurements of the Dark Treatment Box were made. The average value recorded for darkness measurements on the spectroradiometer was 9.0E-05 ± 3.3E-04 . (n= 4000 data points, 5 each at even numbered nanometers between 300 - 1100). Log graphs plotted to accentuate irradiance in the tail sections of the curves show that the 880 nm LEDs began to radiate at 770 nm (Figure 4). When passed through an IR filter the 880 nm source yielded measurable radiation at 800 nm. Also shown in the log graph, the 935 nm LEDs began to radiate at 880 nm. When passed through an IR filter, the 935 nm source yielded measurable radiation at 890 nm.

Through the spectroradiometer readings, it was noted that the LEDs which were marketed to have peak wavelengths of 880 and 935 nm had peak wavelengths higher than their marketed

values. For simplicity throughout this paper they are referred to by their catalog values of 880 nm and 935 nm LEDs, however, average measured peak wavelengths were actually 916 nm and 958 nm, respectively. Average band width at half-height for the 880 nm LEDs was 94 nm. Average band width at half-height for the 935 nm LEDs was 50 nm.

To further characterize the IR filterk, measurements were made using the 880 nm LEDs as a source. Without the IR filter, the 880 nm LEDs yielded an average of 0.050 μmol m^{-2} s^{-1} in the (far-red) region from 770-780 nm. This was reduced when the IR filter was used in combination with the 880 nm LEDs to 0.004 μmol m^{-2} s^{-1} in the (far-red) region from 770-780 nm. The IR filters only slightly changed the starting wavelengths for light emission The filter did little to change the light quality, but did significantly decrease the light quantity. This effectively reduced the measurable light in the tail cut-off regions, thus slightly increasing the starting measurable emission wavelength.

Discussion and Conclusions

The results of this study show that long duration exposure of *Avena sativa* L. Seger to IR LED radiation is perceptible to oat seedlings. The plants were not affected phototropically (i.e., the IR LED irradiated plants did not bend towards the LEDs); however, the IR LED irradiated seedlings' mesocotyl tissue displayed an orthogravitropic response while dark-grown plants exhibited a diagravitropic response.

Suppression of mesocotyl tissue development and advanced tissue unfurling, known to be phytochrome mediated responses (4, 6) were observed in the IR LED-treated seedlings. Thus, if the phytochrome system is being activated by some component of the IR LED/IR filter system, then either a minute amount of far-red light is emanating from the system; causing a very low fluence response, or the phytochrome may be being activated in the near infrared region as well. The spectroradiometer data shows that the 880 nm LEDs may yield minute quantities of far-red, however, no measurable far-red light was detected from the 935 nm LED source. This supports the latter hypothesis, that is, phytochrome may be being activated in the near infrared region by the 935 nm LEDs, and both the far-red and near infra-red regions by the 880 nm LEDs. This is consistant with the findings of Liscum and Hangarter (8), suggesting that absorption by the P_{fr} form of phytochrome and the subsequent photoconversion to the P_r form is responsible for the observed gravitropic response.

The mesocotyl tissue's orthogravitropic response was activated by both the 880 and 935

LEDs, however, the response appeared more pronounced in the 880 treated seedlings, demonstrating an increase in response as the wavelength of the light gets closer to the visible.

Although the precise wavelengths of radiation causing the effect are yet to be identified, the observation is important for investigators using IR LEDs as a study tool for dark-viewing.

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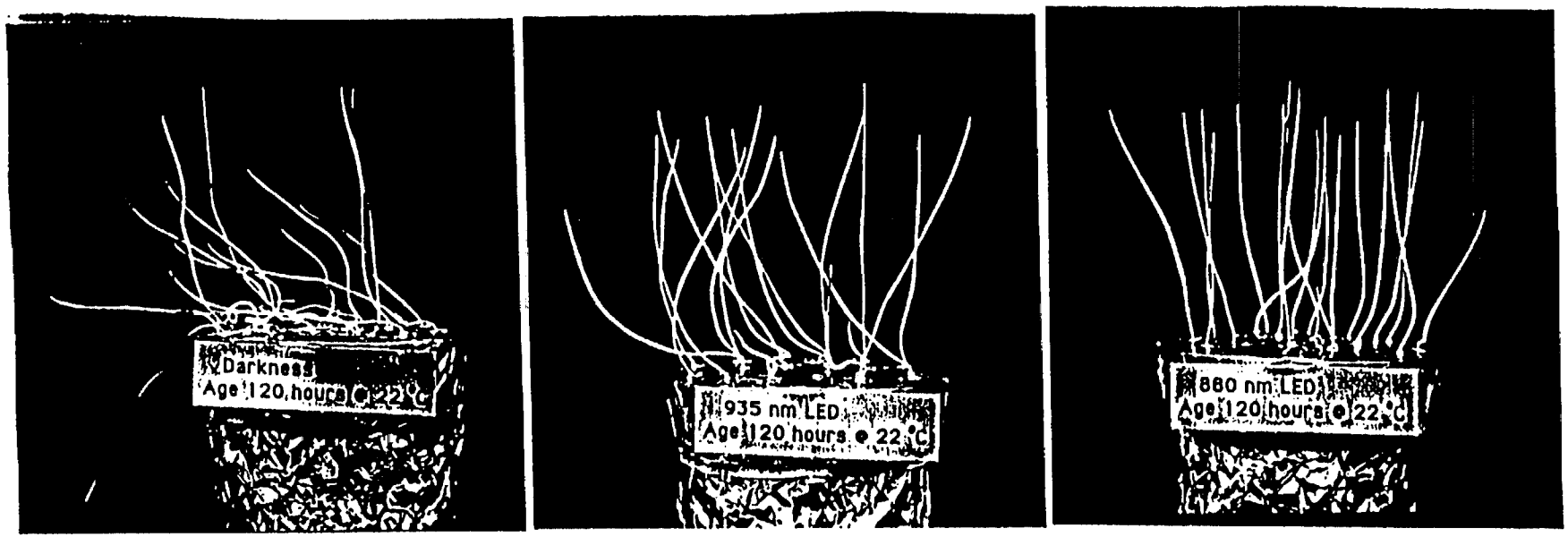
TABLE I. Plant growth and angle data. Students' t-test groupings: Averages ± Standard Errors shown. Means of each column followed by the same letter are not significantly different at the 0.05 level. Test series #1 and #2 data statistically analyzed separately.

Treatment	Total Height	Leaf length	Percent mesocotyl	Average seedling tip-to-base angle (degrees)
First set of Experiments (Plant age 120 hours)				
	(mm)	(mm)	(%)	
Dark	100.1 ± 2.2 x	0 x	54.6 ± 1.4 (x)	146.4 ± 5.8 x
935 nm	98.4 ± 2.8 x	0 x	35.6 ± 1.1 (y)	86.0 ± 3.5 y
880 nm	88.1 ± 2.4 y	7.4 ± 1.3 y	17.7 ± 0.9 (z)	91.8 ± 2.6 y
Second set of Experiments (Plant age 84 hours)				
	(mm)	(mm)	(%)	
Dark	37.6 ± 2.1 a	0 a	60.6 ± 0.5 (a)	159.8 ± 4.1 (a)
935 nm LED + IR Filter	32.6 ± 1.8 b	0 a	48.9 ± 1.6 (b)	127.3 ± 5.7 (b)



(a) (b) (c)

FIGURE 1. *Avena sativa* seedlings, age 120 hours, grown at 22 .5 ^{o}C under continuous (a) darkness, (b) 935 nm IR LED irradiation, and (c) 880 nm IR LED irradiation

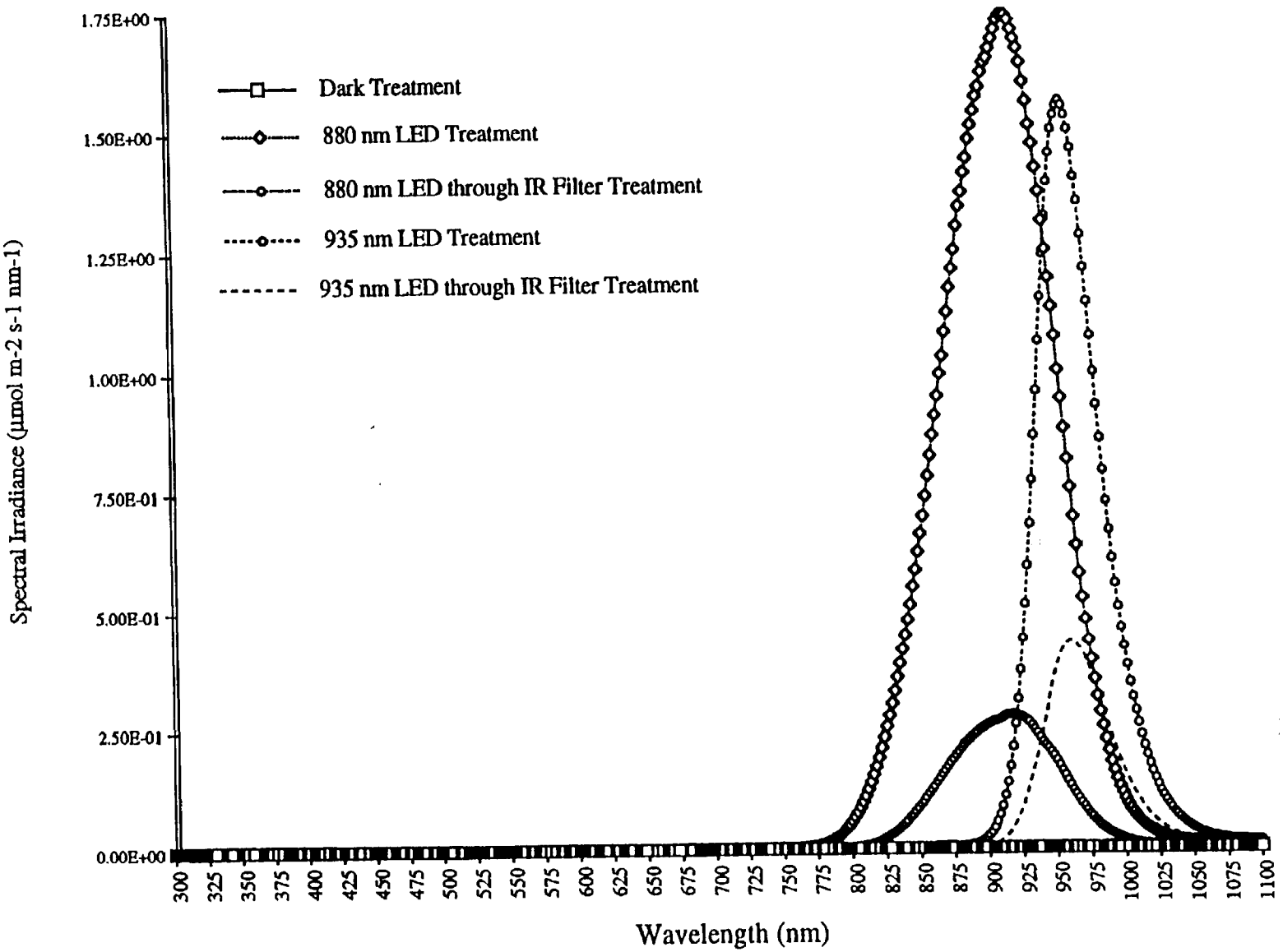


FIGURE 2. Linear analysis of spectral output from spectroradiometer

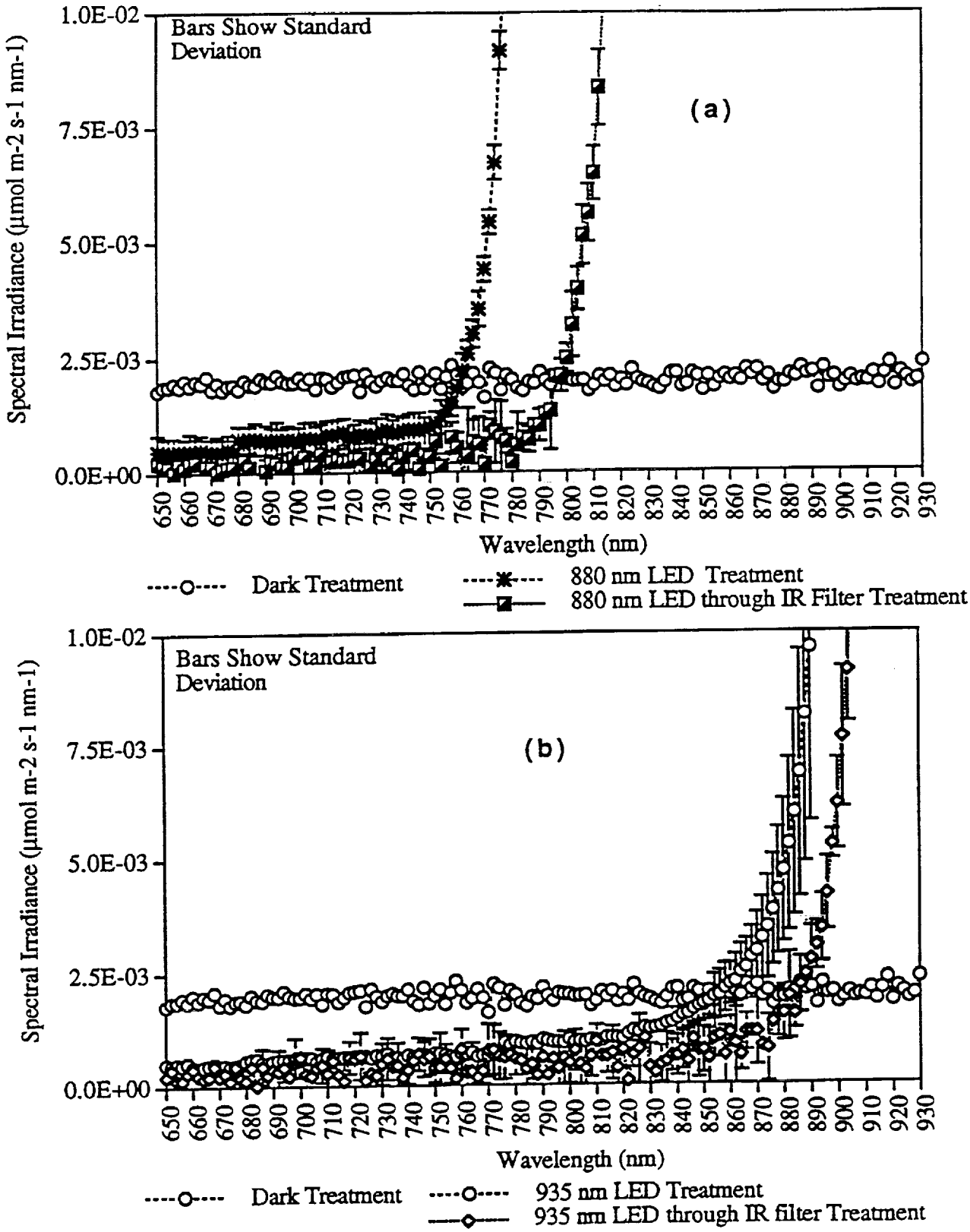


FIGURE 3. Linear graphs from spectroradiometer, close-up of tail regions for (a) 880 nm LEDs (with and without IR Filter) and (b) 935 nm LEDs (with and without IR Filter)

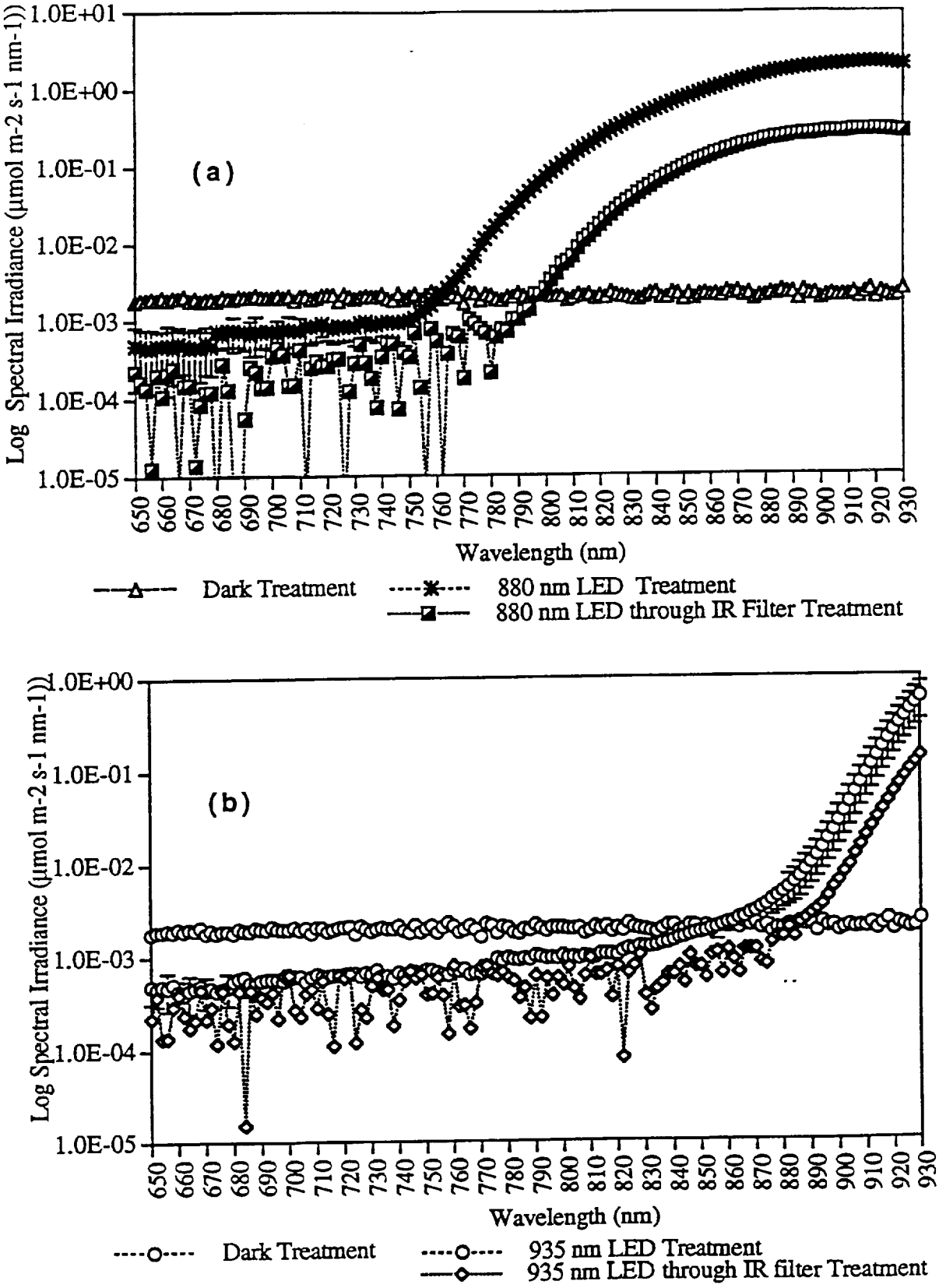


FIGURE 4. Log scale graphs from spectroradiometer, close-up of tail regions for (a) 880 nm LEDs (with and without IR Filter) and (b) 935 nm LEDs (with and without IR Filter)