Biochemical Differences Between Embryonic Axes from Green and Sun-bleached Lima Bean Seeds: Synthesis of Carbohydrates, Proteins, and Lipids

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Abstract. Green and sun-bleached seeds of cultivars 'G 2' and 'Green Fordhook 861' lima beans (Phaseolus lunatus L.) were analyzed for total chlorophyll in whole seeds, and for soluble protein and rates of synthesis of carbohydrates, proteins, and lipids in excised embryonic axes. Bleached seeds were lower in total chlorophyll than green seeds. Embryonic axes from bleached seeds synthesized less carbohydrates and proteins, but more lipids than embryonic axes from green seeds. When held in water for 3 hours, embryonic axes from 'G 2' lima beans had about 65% more soluble protein, and respired 20% faster than embryonic axes from 'Green Fordhook 861'. Whole 'G 2' lima bean seeds also emerged faster than 'Green Fordhook 861' seeds. In each cultivar, green seeds had a higher percent emergence than bleached seeds.

Bleaching of the green color in seeds is a serious problem in many species (7, 12, 14). In lima bean seeds with green cotyledons, exposure of pods to sunlight during seed maturation and curing stimulates the destruction of the green pigment (chlorophyll) and leads to bleached seed. Bleached beans in processed products reduce the quality of the product in proportion to occurrence (13), and, upon planting, they produce plants with lower vigor than plants produced from green seeds under the same conditions (8, 12, 16). Bleached lima bean seeds often exhibit higher frequency of mechanical injury (14) and lower germinability and seedling growth than green seeds (12, 14). Bleached seeds also have less tolerance to low temperatures during the imbibition stage of germination (8) and lower respiratory rates than green seeds during germination (16).

Studies on green tissues suggest that bleaching is a temperature-independent photo-oxidative process (17, 18). The rate at which sunlight bleaches tissues depends on (a) light quality, intensity, and duration, and (b) thickness of the zone which contains the chloroplasts. The sequence of changes during photo-bleaching, as revealed by in situ studies on chloroplasts of leaves from several species of plants, appears to be similar for all species (17). Destruction of chloroplasts requires 50 klux or 5 times the intensity required for photosynthetic saturation. All visible radiation above 5 times the photosynthetic saturation induces bleaching of plant tissue, and relative bleaching effectiveness is proportional to absorption of radiation of a particular wavelength by the photosynthetic pigment. Thus, blue light which is absorbed most, causes most bleaching and green light, which is absorbed least, causes least bleaching.

This study reports the reduction in chlorophyll content of bleached seeds and the capacity of excised lima bean embryonic axes from green and bleached seeds to synthesize carbohydrates, proteins, and lipids during early hours of germination. Differences in synthetic capacity of embryonic axes from green and bleached seeds are also discussed with respect to differences in emergence time and total emergence of seedlings from seeds grown in the greenhouse.

Materials and Methods

Materials. Seeds from 2 cultivars of lima beans with green cotyledons 'G 2' and 'Green Fordhook 861' were used. They were produced in California in 1968 and obtained from Ben Fish and Company of California. On arrival, the seeds were hand sorted into 3 color categories: green, intermediate, and bleached (white). Green and bleached seeds with sound seed coats were placed in Mason jars and stored at 5°C until planting. Seeds from the intermediate group and all seeds with mechanically injured seed coats were discarded. The whole seeds were used for determining emergence, moisture, and chlorophyll content, whereas excised embryonic axes were used to determine respiratory rates and carbohydrate, protein, and lipid synthesis.

Seeding emergence. Emergence tests in the greenhouse were made by planting 400 seeds for each treatment in 45 x 35 cm trays (75 seeds in each of 5 trays and 25 seeds in one tray) containing sterilized soil. The seeds were treated with Thiram before planting. Greenhouse temperature ranged from 24 to 30°C. Normal seedlings (those with at least one cotyledon, long healthy hypocotyl, and well developed roots) were counted up to 14 days from planting.

Chlorophyll determination. Chlorophyll was extracted according to the method described by Vishniac (11). Whole dry seeds were ground in a Wiley Mill. Six-gram samples of the hand sorted material were extracted 7 times, each time in 5 ml absolute methanol under dim light at room temperature. The combined extracts were passed through Whatman No. 1 filter paper and total chlorophyll was determined spectrophotometrically by measuring the optical density of the solution at 650 and 665 nm.

Preparing excised embryonic axes. Excised embryonic axes were prepared by scoring the seed coat of the dry seed with a razor blade along the side opposite to attachment of embryonic axes to cotyledons to permit fast uniform entry of water into cotyledons. The seeds were then held for 2 hr in sterilized water containing 20 µg/ml penicillin-G and streptomycin sulfate at 25°C. Excising the embryonic axes was more easily accomplished at the end of 2-hr imbibition than before imbibition. Excised embryonic axes were kept moist in a Petri dish until incubated in various solutions. Incubation began 3 hr from the start of imbibition.

Incorporation of label: The ability of excised embryonic axes to synthesize carbohydrates, lipids, and proteins was determined by incubating embryonic axes for 2 hr in solutions containing 14C-labeled glucose, acetate, and leucine, respectively, in the presence of penicillin-G and streptomycin sulfate (20 µg/ml of each). Details of the incubation and the subsequent extraction
of newly synthesized carbohydrates and proteins have been reported earlier (2). Media and duration of incubation are described in each table. Water-soluble proteins were precipitated in 10% cold perchloric acid (PCA), washed 3 times in PCA, and solubilized in 0.3 N KOH (2), then determined by the method of Lowry et al. (6).

Lipid synthesis was determined by incubating excised embryonic axes in 14C-glucose or 14C-2-acetate, collecting the 14CO2 evolved during incubation, and extracting the tissue using procedures described by Entenman (4) with the following modifications. The tissue was ground in 10% cold trichloroacetic acid (TCA) in a mortar, the slurry was centrifuged at 3000 g for 10 min, and the supernatant was decanted. The precipitate was washed 4 times in cold 10% TCA, and centrifuged as before. The washed precipitate, which contained the proteins and 90% of total tissue lipid, was extracted for 40 min in boiling petroleum ether (35 ml per 20 embryonic axes) and the petroleum ether-soluble fraction which contained the lipids was saved. This step was repeated 5 times to insure complete extraction of lipids. The combination of petroleum ether fractions was evaporated to a small volume and the radioactivity was determined by liquid scintillation and expressed in disintegrations per minute (dpm) (2). This procedure allowed approximately 90% recovery of total lipids. The other 10%, which was found in the cold TCA-soluble fraction, was extracted by neutralizing the TCA fraction with 14 N KOH to pH 7.0, then extracting 3 times in petroleum ether at room temperature in a separatory funnel. The lipid content of the cold TCA-insoluble fraction plus that from the TCA-soluble fraction is referred to as “total lipid”.

Incorporation of labeled C into carbohydrates and proteins in the TCA-insoluble and soluble fractions after the extraction of lipids was determined. The TCA-insoluble fraction was washed once with 80% ethanol, centrifuged, and the radioactivity of the precipitate was measured. Carbohydrates in the TCA-insoluble fraction (lipid removed) were determined by precipitating in 80% ethanol at 0°C overnight. The precipitate was washed 3 times in 80% ethanol. The sum of the two ethanol-insoluble fractions is referred to as “total carbohydrates and proteins”.

Respiration. Oxygen uptake, CO2 production, and respiratory quotient (RQ) values were determined manometrically on excised embryonic axes to gain more information about shifts in intermediary metabolism of embryonic axes due to bleaching (2).

Moisture determination. Seed moisture was determined on whole seeds before planting; 15 to 17-g samples (6 replicates per treatment) were dried in an oven over forced hot air for 60 hr at 103°C ± 3°C (3).

Results

Emergence time and total emergence. The data on emergence time and total emergence of normal seedlings from green and bleached seeds of ‘G 2’ and ‘Green Fordhook 861’ suggest that green seeds of both cultivars had higher total emergence than bleached seeds (Fig. 1). Seedlings of ‘G 2’ emerged 1 to 2 days earlier than those of ‘Green Fordhook 861’. Emergence time for seedlings, however, did not differ between green and bleached seeds of the same cultivar.

Seed size and moisture. Bleached seeds from ‘G 2’ and ‘Green Fordhook 861’ were about 15% and 8% larger than green seeds, respectively (Table 1). Furthermore, the dry weight per seed of ‘Green Fordhook 861’ seeds was about 2.5 times that of the ‘G 2’ seed. Moisture contents of green and bleached ‘G 2’ seeds were 11.2 and 11.1%, respectively, and those of green and bleached ‘Green Fordhook 861’ were 11.6 and 13.0%, respectively.

Reduction in seed chlorophyll. Green seeds of ‘G 2’ and ‘Green Fordhook 861’ cultivars contain approximately equal amounts of chlorophyll (Table 2). This pigment is located both in the embryonic axes and the cotyledons. Green leaves contain 40 to 45 times as much chlorophyll as green lima bean seeds per unit of fresh weight (5). Bleached seeds lose over 90% of the chlorophyll and most of this loss occurs during maturation and drying while the seeds are still inside the pods.

Biosynthetic activity of excised embryonic axes. When excised embryonic axes from green and bleached seeds were held in water for 3 hr, then incubated in 14C-glucose, 14C-2-acetate or 14C-leucine, embryonic axes from green seeds incorporated 70 to 100% more labeled glucose and 30% more labeled acetate into carbohydrates and proteins than embryonic axes from bleached seeds (Tables 3, 4). The percent of 14C-glucose incorporated into lipids was the same in embryonic...
Embryonic axes from green and bleached seeds differed greatly in the rates of incorporation of \(^{14}\text{C}\)-glucose into various macromolecules but agreed in the relative distribution of incorporated glucose (Table 5). The relative distribution of \(^{14}\text{C}\) among the various fractions of both types of embryonic axes was about 65% in hemicellulose and starch, 11% in cellulose, 10% in protein, 10% in CO\(_2\), and about 2% in water-soluble carbohydrates. Largest differences in incorporation rates of \(^{14}\text{C}\)-glucose between embryos from green and bleached seeds were in the hemicellulose-starch and cellulose fractions. Smallest differences were in the water-soluble carbohydrates and in CO\(_2\).

The incorporation rate of \(^{14}\text{C}\)-leucine into proteins, as determined from the specific activity of water-soluble proteins, was 18% to 29% higher in embryonic axes from green seeds than from those bleached (Table 6). Embryonic axes from green and bleached seeds of the same cultivar contained equal amounts of soluble proteins (Table 7). Soluble protein (mg per g dry weight) in embryonic axes of 'G 2' was approximately 65% higher than that in embryonic axes of 'Green Fordhook 861' (Table 7).

There was no difference in O\(_2\) uptake of CO\(_2\) evolution by embryonic axes from green and bleached seeds of the same cultivar during the period when rates of synthesis of carbohydrates, proteins, and lipids were measured (Table 8). In contrast, embryonic axes from 'G 2' seeds (green and bleached) consumed 25% more O\(_2\) and produced 25% more CO\(_2\) than those of 'Green Fordhook 861' on a fresh weight basis (Table 8).
Table 6. Incorporation of 14C-leucine into proteins by embryonic axes of green and bleached lima bean seeds.

<table>
<thead>
<tr>
<th>Cultivar and color</th>
<th>Water soluble proteins</th>
<th>Cold water soluble residue</th>
<th>PCA soluble</th>
<th>Total uptake</th>
<th>% Incorporated into soluble proteins</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm/20 embryonic axes</td>
<td>dpm/mg Protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G 2 green</td>
<td>30700</td>
<td>1720</td>
<td>50780</td>
<td>83190</td>
<td>36.9 b</td>
<td>1030 b</td>
</tr>
<tr>
<td>G 2 bleached</td>
<td>26110</td>
<td>2940</td>
<td>56510</td>
<td>85560</td>
<td>30.6 a</td>
<td>801 a</td>
</tr>
<tr>
<td>Fordhook 861 green</td>
<td>39700</td>
<td>2380</td>
<td>66030</td>
<td>108120</td>
<td>36.8 b</td>
<td>1039 b</td>
</tr>
<tr>
<td>Fordhook 861 bleached</td>
<td>33990</td>
<td>2350</td>
<td>66910</td>
<td>103260</td>
<td>31.7 ab</td>
<td>929 ab</td>
</tr>
</tbody>
</table>

*Embryonic axes were incubated for 3 hr in sterile water containing 20 μg/ml each of penicillin-G and streptomycin sulfate, incubated for 2 hr at 25°C in 10^-3 M 14C-leucine (1 μc/ml), washed 4 times in 10^-3 M leucine and extracted as described in reference 1.

Table 7. Dry weights and soluble protein content of embryonic axes from green and bleached lima bean seeds.

<table>
<thead>
<tr>
<th>Cultivar and color</th>
<th>Dry wt/100 embryonic axes</th>
<th>Soluble proteins/g embryonic axes</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td></td>
</tr>
<tr>
<td>G 2</td>
<td>505 a</td>
<td>295 b</td>
<td></td>
</tr>
<tr>
<td>G 2 bleached</td>
<td>572 a</td>
<td>285 b</td>
<td></td>
</tr>
<tr>
<td>Fordhook 861 green</td>
<td>1072 b</td>
<td>178 a</td>
<td></td>
</tr>
<tr>
<td>Fordhook 861 bleached</td>
<td>1076 b</td>
<td>170 a</td>
<td></td>
</tr>
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</table>

*Each figure is the mean of 2 experiments with 4 replicates in each. Letters following means indicate Duncan’s Multiple Range Test values at 1% significance level. Comparable means followed by no letters in common are significantly different.

Table 8. Oxygen uptake, CO2 production, and respiratory quotients (RQ) of lima bean embryonic axes from green and bleached seeds.

<table>
<thead>
<tr>
<th>Cultivar and color</th>
<th>O2 uptake (μl/hr)</th>
<th>CO2 evolution (μl/hr)</th>
<th>RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G 2</td>
<td>272 b</td>
<td>222 b</td>
<td>0.82 a</td>
</tr>
<tr>
<td>G 2 bleached</td>
<td>262 b</td>
<td>216 ab</td>
<td>0.82 a</td>
</tr>
<tr>
<td>Fordhook 861 green</td>
<td>208 a</td>
<td>178 ab</td>
<td>0.86 a</td>
</tr>
<tr>
<td>Fordhook 861 bleached</td>
<td>204 a</td>
<td>172 a</td>
<td>0.84 a</td>
</tr>
</tbody>
</table>

*Each figure is the mean of 2 experiments with 4 replicates in each. Letters following means indicate Duncan’s Multiple Range Test values at 1% significance level. Comparable means followed by no letters in common are significantly different.

Discussion

The results on total emergence (Fig. 1) confirm earlier findings that bleached seeds have lower germinability than green seeds (12, 14). Differences in emergence time between green and bleached seeds of the same cultivar are small and may, in part, be related to differences in size, physiological maturity, and moisture content of seeds (8, 9, 15). Thus, the main difference between green and bleached seeds of the same cultivar is the lower total emergence of bleached seeds in comparison to green seeds.

Although loss of the green color from lima bean seeds has been a reliable index of vigor reduction (14), no direct relationship between loss of vigor and decomposition of chlorophyll has been proven. Likewise, the effects of sunlight on changes in cellular organelles and metabolic processes have not been investigated. It is therefore reasonable to suggest that damage by sunlight is not necessarily due to decomposition of chlorophyll, but instead, may be due to certain vital metabolic processes that may be equally susceptible to damage by light. The reduced rates of carbohydrate and protein synthesis in embryonic axes from bleached seeds support this conclusion. Rates of synthesis of carbohydrates and proteins have been proposed as indices for measuring seed vigor because they were affected by treatments that reduced vigor, such as heat and unfavorable storage conditions (1). Unlike protein and carbohydrate synthesis, lipid synthesis is not closely associated with vigor. The capacity of sun-bleached lima bean embryonic axes to synthesize more lipids is somewhat analogous to that of mature fruits. Synthesis of lipids in apples and many other fruits increases as the fruit approaches senescence (10).

That respiratory metabolism was neither qualitatively nor quantitatively affected by sunlight is demonstrated by the similarity in O2 uptake, CO2 evolution, and RQs of imbibed embryonic axes from green and bleached seeds of the 2 cultivars. The data suggest that both types of embryonic axes are utilizing stored carbohydrates and lipids in almost equal amounts during the early hours of imbibition (5). Contrary to these findings on excised embryonic axes, reduced respiratory rates in bleached lima bean seeds (16) 2 hr after imbibition have been reported.

When embryonic axes from green and bleached seeds were incubated in 14C-labeled acetate, differences in their rates of incorporating the label into carbohydrates were consistently smaller than when they were incubated in 14C-glucose (Table 3, 4). That the metabolic pathway from glucose to carbohydrates is more direct and involves fewer reactions than the pathway from acetate to carbohydrates explains the difference in incorporation of the two substrates into the same kind of polymer by embryonic axes from green and bleached seeds.

The higher soluble protein content of 'G 2', as compared to 'Green Fordhook 861' (Table 7) is important because of the role soluble proteins play in biological systems. Some of these proteins are enzymes which regulate the growth processes. The high content of soluble proteins in 'G 2' might explain, in part, the earlier emergence and high total emergence of normal seedlings exhibited by this cultivar in comparison with 'Green Fordhook 861' (Fig. 1). The smaller size of 'G 2' seeds and possible difference in rate of water uptake between the 2 cultivars also could affect emergence. Furthermore, the higher respiration of 'G 2' embryonic axes (Table 8) also supports the observation that 'G 2' seeds are more vigorous than those of 'Green Fordhook 861'. Further tests using more cultivars should be conducted to prove or disprove the suggestion that vigor is related to high soluble protein content of the embryonic axes.
Growth Responses Induced by Brassins (fatty plant hormones) in Bean Plants

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Abstract. A single application of 10 μg of brassins to a young internode of a 'Pinto' bean plant accelerated growth of the treated internode and of other internodes above and below the treated area. The treated internodes grew longer overall and thicker in some areas than comparable untreated ones. These responses involve both cell elongation and cell division. The treatment also caused marked changes in the vascular anatomy of leaves above the site of application.

Brassins, a new family of plant hormones from rape pollen, were recently described (4). They were reported to be lipids having a glyceride structure. Responses of plants to brassins have not yet been studied in general, since at present only limited amounts of these hormones are available. It was possible, however, to carry out limited experiments to obtain some information about the effects of these substances on young bean plants. Results of greenhouse experiments supplemented observations made during numerous bioassays conducted in a growth room with bean plants over a period of 2 years.

The present report concerns growth responses of bean plants to topical applications of brassins and describes morphological and anatomical changes involved in these responses.

Methods

Ether extracts of rape pollen (Brassica napus L.) were purified by thin-layer chromatography as previously described (4) and applied to young bean plants, Phaseolus vulgaris cv. Pinto, in accordance with the bean second internode method (3). In each experiment, 10 μg of the purified brassins was applied in the fractionated lanolin carrier to each of 4 plants. An equal number of comparable plants were treated in the same manner with the fractionated lanolin alone. Plants in the growth room received about 700 ft-c of light from Slimline Daylite\(^3\) fluorescent tubes for 8 hours daily at a temperature of 72° to 74°F. Plants in the greenhouse were grown under natural light and daylength at 65° to 85°.

Growth in terms of internode length, and in some instances volume, was recorded and obvious responses were noted. Representative parts of stems and leaves from both treated and control plants were harvested, fixed in formalin:acetic acid:alcohol, dehydrated, embedded in Paraplast, sectioned at 10 μ and stained with safranin:fast green (1).

Results

Growth Room: Marked responses of plants treated with the brassins were observed (Fig. 1A). The increase in length of the treated second internode, which is approximately 400 percent in the plant on the right, was sometimes as much as 1000 percent over the average of the control plants. A visible response to a 10-μg treatment was usually detected within 48, and sometimes within 24 hours. Typical comparative rates of internode elongation for treated and control plants are shown in Fig. 2.

Histological studies showed that this overall increase in internode length is due to both cell elongation and cell division. Cells in the basal part of the treated internode undergo...