Interaction of Boron with Components of Nucleic Acid Metabolism in Cotton Ovules Cultured in vitro

Elliott H. Birnbaum, W. Mack Dugger, and Bud C. A. Beasley
Departments of Biology and Plant Sciences, University of California, Riverside, California 92502

ABSTRACT

Cotton (Gossypium hirsutum L.) ovules grown in a defined nutrient medium undergo normal morphogenesis, including fiber production. In identical medium lacking boron, ovules callus and accumulate brown substances. Boron deficiency-like symptoms were induced by 6-azauracil and 6-azauridine in ovules growing in boron-sufficient media. Other nucleoside base analogs either reduced or had no effect on over-all growth, but did not cause typical boron-deficient callus growth of cotton ovules. Orotic acid and uracil counteracted the effects of 6-azauracil. Actinomycin D, fluorodeoxyuridine, and ethidium bromide reduced not only fiber production on ovules growing in boron-sufficient media but also callusing of ovules in boron-deficient media.

Similarities between symptoms of boron deficiency and 6-azauracil injury, and the ability of uracil to suppress both, suggest that boron deficiency symptoms are related to reduced activity in the pyrimidine biosynthetic pathway. Growth inhibition by most nucleoside base analogs tested, actinomycin D, fluorodeoxyuridine, and ethidium bromide, as compared to callusing brought on by boron deficiency and 6-azauracil, indicates that boron deficiency symptoms are not related to a reduction in nucleic acid biosynthesis. Based on this information, a discussion of the possibility that boron deficiency causes reduced synthesis of UDP-glucose is presented.

Research on the role(s) of boron in higher plants suggests its involvement not only with translocation and nucleic acid metabolism, but also with responses to plant growth regulators, phenolic acid biosynthesis, cell wall metabolism, cell maturation and division, and certain enzyme-mediated reactions (11). Direct involvement of boron in only one or a few metabolic processes might have many secondary effects on plant growth; thus, any of the above might be influenced only indirectly by the sufficiency or deficiency of the element.

Changes in nucleic acid levels have been correlated with boron deficiency. Some workers have reported that RNA levels increase during boron deficiency (8–10) while others have observed a decrease in nucleic acid levels (5, 7). Chapman and Jackson (7) found that an early sign of boron deficiency in Phaseolus aureus root tip segments was increased incorporation of radioactive precursors into RNA. Only later, concomitant with increased ribonuclease activity and cessation of elongation, did a reduction in RNA levels occur. They point out that reports of boron deficiency-related reductions in RNA were in grossly deficient tissues (1, 18) and that a much earlier and possibly more direct effect of boron deficiency was increased RNA synthesis (8, 9).

Shkol'nik and Soloviyova (30) reported that deleterious effects of boron deficiency might be eliminated by addition of RNA to the growth medium. Johnson and Albert (19) tested the effects of various nitrogen bases on root growth of tomato plants cultured in boron-sufficient and -deficient liquid media. Their results suggested that thymine, guanine, and cytosine suppressed development of boron deficiency symptoms as reflected in reduced root elongation, lowered RNA content, loss of fluorescence, and browning. On the other hand, 6-AU3 and barbituric acid induced these aberrations in root tips grown in the presence of boron.

Unfertilized cotton (Gossypium hirsutum L.) ovules cultured on completely defined liquid growth medium will produce fiber and mimic in vivo growth of fertilized ovules (although embryos are not produced) if the medium is supplemented with 5 μM IAA, 0.5 μM GA3, and 0.05 μM Kin (3). If boron is omitted from this medium, growth is quite aberrant; little or no fiber is produced, and the inundated and peripheral surfaces of the ovules callus profusely, obliterating any semblance of normal morphology (4). The morphological and physiological processes of cotton fiber growth involve elongation and maturation of single epidermal cells. Ovarial callus proliferation, on the other hand, involves cell division that leads to abnormal structure.

We have reported that development of boron deficiency symptoms in cultured cotton ovules is determined in part by the phytohormones included in the basal growth medium (4). Pro- fuse callusing in the absence of boron occurs only in the presence of GA3. Phytohormones have been shown to affect nucleic acid metabolism (20). Thus, one possible cause of callus production in boron deficiency is altered nucleic acid metabolism. In this paper, we report the effects of nucleoside bases, nucleotides and their analogs, and inhibitors of nucleic acid biosynthesis on growth of unfertilized cotton ovules in boron-sufficient and -deficient media.

MATERIALS AND METHODS

Procedures for flower production, boron-sufficient and boron-deficient ovule culture, and measurement of fiber and ovule growth have been published previously (2–4). Fiber production is expressed in terms of TFU, as determined by the stain–destain method (2), and total mass is expressed as dry wt (mg/ovule).4 Replicated treatments consisted of two to six flasks, each containing 28 to 32 ovules from a single boll (ovary). Experiments were terminated after 14 days of culture. Twentv ovules repre-

1 Present address: Research and Development Authority, Ben-Gurion University of the Negev, P.O. Box 1025, Beer-Sheva, 84110, Israel.

3 Abbreviations: 6-AU: 6-azauracil; Kin: kinetin; 5-FU: 5-fluorouracil; TFU: total fiber units; 2-TU: 2-thiouracil; 5-AU: 5-azauracil; 6-AUR: 6-azauridine; 8-AG: 8-azaguanine; OMP: orotidine monophosphate; FdUrd: fluorodeoxyuridine; TDR: thymidine.

4 Callus tissue adsorbs a small amount of the stain used to determine TFU. Low TFU values are therefore observed for fiberless ovules which have undergone callusing. Thus, treatments which reduce callusing also result in slightly lower TFU values.
sentative of the treatment were selected from each flask for TFU determination. Dry wt was determined by pooling all sets of 20 ovules utilized in any treatment.

Composition of the culture medium was as previously reported (4) and included IAA, GA₃, and 4K (5, 0.5, and 0.05 μM, respectively). Phytohormones and other supplements were added to the autoclaved basal medium by filter sterilization.

**RESULTS**

**Effects of Intermediates in Nucleic Acid Biosynthesis.** In preliminary experiments, the effects of 0.1 mM adenine, guanine, thymine, cytosine, and uracil were determined on cotton ovules cultured in media without boron. Only uracil appeared to partially alleviate boron deficiency symptoms. Concentration studies with uracil showed that 1 mM was most effective in enhancing ovule and fiber development in boron-deficient medium (Fig. 1). In experiments where the medium contained 1 μM boron, adenine, thymine, and cytosine at 1 mM had no effect; uracil, UMP, and perhaps orotic acid and uridine did enhance cotton fiber growth. Guanine was insoluble and not tested (Table I).

Uracil was analyzed for boron contamination by flame spectrography and found to be boron-free. There was variability in response to uracil between experiments, caused perhaps by the variable level of endogenous boron in the ovules, and related to the variable light conditions during growth of the parent cotton plant. There are indications in the literature that light quality and duration influence the responses of plants to boron or the lack of it (14, 17). Although the effect of uracil was variable, inhibition was always recorded at concentrations greater than 1 mM.

The measurements reported in Figure 2 were made on experiments conducted over a shorter period of time and with less variability in total irradiance received by the plants from which the ovaries were harvested. In this series of experiments, the uracil effect is apparent only when there is a small amount of boron in the culture media (>1 μM and <10 μM). In cultures where no exogenous boron was added to the flask, there was no enhancement of cotton fiber production by 1 mM uracil; where there was 10 μM or more boron added to the media there was no significant response brought about by the inclusion of uracil.

![Unfertilized cotton ovules grown for 14 days in (A) medium containing 100 μM boron; (B) boron-free medium; (C) boron-free medium supplemented with 1 μM uracil.](Fig. 1)

**Table I. Effects of nucleosides, uridine, orotic acid and UMP on TFU production of unfertilized cotton ovules cultured for 14 days in boron-deficient (1 μM) nutrient media**

<table>
<thead>
<tr>
<th>Analogue</th>
<th>D+ (100 μM)</th>
<th>D+ (1 μM)</th>
<th>A(3)</th>
<th>C</th>
<th>T</th>
<th>OA</th>
<th>UMP</th>
<th>U</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFU</td>
<td>8.62</td>
<td>1.69</td>
<td>1.05</td>
<td>1.71</td>
<td>1.61</td>
<td>2.55</td>
<td>3.01</td>
<td>2.58</td>
<td>3.13</td>
</tr>
<tr>
<td>U+25(2)</td>
<td>26.52</td>
<td>26.46</td>
<td>30.33</td>
<td>30.16</td>
<td>70.49</td>
<td>70.51</td>
<td>60.39</td>
<td>70.39</td>
<td>60.55</td>
</tr>
</tbody>
</table>

1 μM H₂BO₃.
2 Twice the standard error
3 Abbreviations: A, adenine; T, thymine; C, cytosine; OA, orotic acid; UMP, uridine monophosphate; U, uracil; D, uridine; S, uracil.

**Effects of Uracil and Pyrimidine Analogs.** The influence of the following purine and pyrimidine analogs on cotton ovule growth in boron-sufficient media was determined at concentrations between 0.1 and 100 μM: 5-FU, 2-TU, 5-AU, 6-AU, 6-AUR, and 8-AG. Only with 5-AU was there no inhibition in fiber growth and dry wt production at any of the concentrations used. At certain concentrations, the others inhibited both TFU and dry wt, e.g., 100 μM 5-FU reduced TFU and dry wt, respectively, to 45.5% and 65% of the boron-sufficient control. The same concentration of 2-TU caused ovules to become brown-shrunken and reduced dry wt to 15.1% of the B(+) control. At 1 μM 8-AG, TFU were reduced to 62.7% and dry wt to 81.5% of the B(+) control. Ten μM 8-AG resulted in 15.5% of the B(+) control TFU and 41.1% of the B(+) control dry wt. In contrast to these effects, ovules in boron-free media developed to 19% of the TFU of B(+) ovules but dry wt increased as a consequence of callusing to 178.2%. Reduction of TFU and dry wt by these compounds may indicate that toxicity is caused by their incorporation into nucleic acids (6, 16). In cotton ovules, there is no similarity between boron deficiency symptoms and those of 8-AG poisoning, as was suggested by Školnik and Smirnov (29).

In contrast to the other compounds tested, 6-AU and 6-AUR induced callusing and reduced TFU (Table II), thereby causing the appearance of boron deficiency-like growth. Fiber production was reduced about 40% by 10 μM 6-AUR, and about 97%
by 100 μM. Dry wt accumulation, however, was inhibited only 10% by 10 μM 6-AUR and 43% by 100 μM 6-AUR. Even greater inhibition of fiber production was caused by 6-AU; at 10 μM it reduced TFU to the level of boron deficiency, i.e., to 19% of the control. As with 6-AUR, the effect of 6-AU on dry wt production was much less than on TFU development. The effects of 1 to 10 μM 6-AU on TFU production and dry wt are shown in Figure 3.

An interesting contrast exists between the effects of 6-AU on ovules growing in boron-sufficient (but excess) and boron-deficient media. During ovule growth at 1 and 10 μM B, 6.4 μM 6-AU reduced TFU production about 40% (Table III) while having a lesser effect on dry wt. However, in the absence of exogenous boron, there was not a significant effect on TFU, but the dry wt was reduced by 43%. This, in effect, produced ovules which were normal in appearance although reduced in size. This suggests that 6-AU inhibits growth of the most rapidly developing cells—fiber in the case of ovules in boron-sufficient medium and callus in boron-deficient medium.

**Interactions of 6-Azauracil and Uracil.** Uracil enters the pyrimidine pathway (Fig. 4) by a salvage mechanism through which UMP is synthesized directly from uracil or with uridine as an intermediate. Thus, it might be expected that uracil would overcome 6-AU interference with cotton fiber growth by serving as an alternate precursor in pyrimidine biosynthesis. In Table IV it is shown that 1 μM uracil did partially overcome 6-AU inhibition of TFU production in boron-sufficient medium. The fact that uracil did not reverse 6-AU inhibition of growth to a greater extent might be related to Ross' observation (26) that incorporation of uracil into UDP-glucose was reduced by 6-AU, perhaps because 6-AU increased the rate of uracil catabolism.

**Effects of 6-Azauracil and Orotic Acid.** Synthesis of UMP by OMP decarboxylase is inhibited by 6-AU (26). Orotic acid, which is the precursor of OMP (the substrate of this reaction), had little effect on boron-deficient growth (Table I). When ovules were cultured in boron-sufficient medium with 6 μM 6-AU, 10 μM orotic acid restored TFU to 68% of the control level (Table V). At the same time, dry wt was only 36% that of control ovules, resulting in small ovules of normal appearance. If only IAA was included in the growth medium rather than the usual combination of IAA, GA₃, and Kin, 6 μM 6-AU inhibited normal growth. Orotic acid (0.1 μM) completely prevented this inhibition, but greater amounts of orotic acid were inhibitory (Table V). These data support information that the effect of 6-AU is via competition with substrates in the pyrimidine biosynthetic pathway. Orotic acid has the ability to prevent 6-AU inhibition of growth and to reach inhibitory levels at lower concentrations in ovules cultured in the presence of only IAA than in ovules cultured with both IAA and GA₃. This suggests, as do the differences in morphology in boron-deficient culture, that boron might normally promote IAA-mediated processes, whereas its absence might allow GA₃-mediated processes to proceed.

**Effects of Actionomycin D.** With the exceptions of 6-AU and 6-AUR, the analogs heretofore discussed are incorporated into

---

**Tables**

| Table III. Effects of 6-Azauracil on TFU and Dry Weight Production of Unfertilized Cotton Ovules Cultured for 14 Days In Boron-sufficient (100 μM) Nutrient Media.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TFU</th>
<th>Dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM 6-AU</td>
<td>5.90 ± 0.67</td>
<td>2.6 ± 0.50</td>
</tr>
<tr>
<td>1 μM 6-AU</td>
<td>2.6 ± 0.50</td>
<td>2.6 ± 0.50</td>
</tr>
<tr>
<td>10 μM 6-AU</td>
<td>0.67 ± 0.50</td>
<td>1.5 ± 0.50</td>
</tr>
</tbody>
</table>

*Twice the standard error

| Table IV. Effects of 6-Azauracil and Uracil Plus 6-Azauracil on TFU and Dry Weight Production of Unfertilized Cotton Ovules Cultured for 14 Days In Boron-sufficient (100 μM) Medium.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TFU</th>
<th>Dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM 6-AU</td>
<td>5.90 ± 0.67</td>
<td>2.6 ± 0.50</td>
</tr>
<tr>
<td>1 μM 6-AU</td>
<td>2.6 ± 0.50</td>
<td>2.6 ± 0.50</td>
</tr>
<tr>
<td>10 μM 6-AU</td>
<td>0.67 ± 0.50</td>
<td>1.5 ± 0.50</td>
</tr>
</tbody>
</table>

*Twice the standard error

| Table V. Effects of 6-Azauracil and 6-Azauracil-Orotic Acid Combinations on TFU and Dry Weight Production of Unfertilized Cotton Ovules Grown 14 Days In Nutrient Media Containing either IAA, GA₃, and Kin or only IAA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TFU</th>
<th>Dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 μM 6-AU</td>
<td>5.90 ± 0.67</td>
<td>2.6 ± 0.50</td>
</tr>
<tr>
<td>1 μM 6-AU</td>
<td>2.6 ± 0.50</td>
<td>2.6 ± 0.50</td>
</tr>
<tr>
<td>10 μM 6-AU</td>
<td>0.67 ± 0.50</td>
<td>1.5 ± 0.50</td>
</tr>
</tbody>
</table>

*Twice the standard error
RNA (6, 27) and probably limit growth by producing nonfunctional or aberrant RNAs. Both 6-AU and 6-AUR interfere with the pyrimidine biosynthetic pathway and caused an effect in cotton ovules closely resembling boron deficiency. It was of interest to determine whether actinomycin D, an inhibitor of RNA synthesis, would limit growth as did most of the other analogs, or whether it might cause aberrant (boron deficiency-like) growth. Actinomycin D, 2 and 20 \( \mu \)g/ml, was tested on ovules growing in boron-sufficient medium. At 2 \( \mu \)g/ml, the TFU level was about 4% that of the control. With 20 \( \mu \)g/ml, ovules enlarged slightly, produced no fiber, and remained white except for a small area on the inner concave surface which browned (data not shown). Ovules were transferred from boron-sufficient or -deficient media into identical media containing 2 \( \mu \)g/ml actinomycin D on various days after initiation of culture (Fig. 5). When transferred on day 5, ovules from boron-sufficient medium produced 37% as much fiber as the control; when transferred on day 2, fiber production was 30% of the control. In boron-deficient medium, ovules transferred to actinomycin D medium on day 5 produced about 29% of the B(--)(-) control TFU, and 51% of the dry wt at the end of 2 weeks in culture. Those transferred on day 2 produced 23% of the B(--)(-) control TFU, and only 29% of the dry wt. Thus, RNA synthesis appears to be necessary for fiber development and normal morphogenesis, as well as for callus growth induced when boron was absent. RNA synthesis appears to occur at all stages of the 14-day culture period.

**Inhibitors of DNA Synthesis.** In order to gain insight into possible differences in DNA composition of normal and boron-deficient tissues, two inhibitors of DNA synthesis, FUdR and ethidium bromide, were tested.

**FUdR.** FUdR interferes with all DNA synthesis by functioning as an alternate substrate for TMP synthetase (ref. 6 and Fig. 4). In the presence of 100 \( \mu \)M boron, 1 and 10 \( \mu \)M FUdR reduced TFU production to the level of boron deficiency (Table VI), however, unlike boron-deficient growth, FUdR reduced dry wt as well. Thus, it appears that both fiber development and ovular growth require DNA synthesis. When ovules were grown in boron-deficient medium, 1 and 10 \( \mu \)M FUdR had a lesser effect on fiber production than when the ovules were grown in boron-sufficient medium. In both cases, dry wt was decreased by FUdR (Table VI). Such ovaries resembled B(+) ovaries grown with FUdR, as well as B(--) ovaries cultured with IAA but no GA. In other words, inulated surfaces neither callused nor produced fiber, and the upper surfaces were covered by sparse fiber growth.

Thymine had no effect on boron-deficient cotton ovule growth (Table III). When 10 \( \mu \)M TdR was added to media in which fiber development (in the presence of boron [Table VI and Fig. 6]) or callus development (in the absence of boron [Table VI]) would have been prevented by 1 \( \mu \)M FUdR, growth occurred as though FUdR had not been included. Thymine and TdR are precursors of TMP via a salvage pathway which bypasses TMP synthetase (ref. 6 and Fig. 4). Reversal of the FUdR effect by TdR is supportive of FUdR's proposed site of action. (Inasmuch as uptake studies were not made, the possibility of competition between FUdR and TdR for transport into cells cannot be ruled out as at least a partial cause of the reversal.) FUdR prevented callusing in a boron-deficient medium as well as fiber development in boron-sufficient medium. This indicates that callusing as an alternate growth form is not a result of insufficient precursor material for pyrimidine biosynthesis, and indeed is as dependent on pyrimidine and DNA synthesis as normal growth.

**Effects of Ethidium Bromide.** In mammalian cells, ethidium bromide inhibits mitochondrial associated RNA synthesis by binding to DNA, and at low concentrations it does not inhibit nuclear DNA synthesis (23). The effects of ethidium bromide on cotton ovule morphogenesis were similar to those of FUdR. In

```
<table>
<thead>
<tr>
<th>Table VI. Effects of Fluorodeoxyuridine (FUdR) Concentration on Fiber (TFU) and Dry Weight Production of Unfertilized Cotton Ovule Cultured in Boron-Sufficient and Deficient Nutrient Media for 14 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B(+)</strong></td>
</tr>
<tr>
<td>FUdR</td>
</tr>
<tr>
<td>TFU</td>
</tr>
<tr>
<td>DFUE(d)</td>
</tr>
</tbody>
</table>

Dry wt

| mg/ovule | 3.4 | 4.4 | 4.3 | 1.4 | 5.7 | 4.1 | 1.5 | 1.3 |

| Tw00 \( \mu \) M NH$_3$ | b) 0 \( \mu \)M NA$_2$ | c) Twice the standard error |

Fig. 5. Effects of actinomycin D (2 \( \mu \)g/ml) on TFU and dry wt production of unfertilized cotton ovules in boron-sufficient (100 \( \mu \)M) and -deficient nutrient media. Ovules were cultured 14, 5, 2, or 0 days in boron-sufficient or -deficient media prior to transfer to identical medium containing actinomycin D for the remainder of the 14-day culture period.

Fig. 6. Effects of fluorodeoxyuridine (FUdR) and thymidine (TdR) combinations on TFU and dry wt production of unfertilized cotton ovules cultured for 14 days in boron-sufficient (100 \( \mu \)M) nutrient media.
```
the presence of boron (Table VII), ethidium bromide reduced fiber and dry wt production; in the absence of boron, it prevented callusing. In both cases, 1 μM ethidium bromide caused ovules to be reduced in size with fiber on the upper surface but none on the inundated surface. Both 10 and 100 μM ethidium bromide reduced ovular growth still further and essentially eliminated fiber production.

**DISCUSSION**

The over-all reduction in growth of boron-deficient ovules affected by inhibitors of RNA and DNA synthesis shows that for callusing to occur, nucleic acid biosynthesis is necessary. Both 6-AU and 6-AUR are converted to 6-AUR monophosphate which interacts with OMP-decarboxylase (27, 32), thereby reducing synthesis of UMP and, consequently, synthesis of nucleic acid pyrimidines and/or UDP-glucose (refs. 23, 26, and Fig. 4). Callus growth on cotton ovules in media containing 6-AU, 6-AUR, and in boron-deficient medium indicated that their effects are not due to insufficient nucleic acid biosynthesis. One possible hypothesis to explain the results of this study is that during boron deficiency a reduction in UDP-glucose pyrophosphorylase activity and subsequent synthesis of UDP-glucose occurs. UDP-glucose is a likely precursor of cellulose and a precursor of other UDP-sugars involved in cell wall composition (e.g. in elongating and maturing fibers). UDP-glucose pyrophosphorylase appears to play a key role in morphogenesis of the slime mold, Dictyostelium discoideum, increasing in activity during the period of fruiting body formation by aggregated amoeboid cells (15, 24). With respect to higher plants, evidence in the literature lends support to the hypothesis that a critical function of boron is in the activity of UDPG pyrophosphorylase. In cocklebur leaf discs incubated with 14C-uracil and 6-AU, the most significant effect of 6-AU was reduction of incorporation of label into UDP-glucose by more than 50% (26). Similarly, in bean roots, boron deficiency resulted in accumulation of UTP and decline in the level of UDP-glucose (ref. 31 and personal communications with the author). Dugger and Humphreys (12) earlier reported that in vitro activity of UDP-glucose pyrophosphorylase was stimulated when boron was added to the reaction mixture.

A number of reports connect boron to various phases of carbohydrate and nucleotide metabolism (11). Lee and Aronoff (21) found that phenol biosynthesis is limited by a borate-6-P-glucanate complex which inhibits 6-P-glucanate dehydrogenase activity. In the absence of boron, greater enzyme activity results in elevated phenol biosynthesis. Phosphoglucomutase activity (22) and starch biosynthesis (13, 28) have also been reported to be inhibited by boron. Regulation of any of these reactions by boron might influence glucose-1-P levels and, thereby, UDPG synthesis and the direction of UTP metabolism.

Although there is evidence of roles for boron in activities of several plant enzymes (11, 12, 14), we favor the hypothesis that boron plays a major role in the development of cotton ovules by regulating UDPG synthesis. If this is so, glucose-1-P would accumulate during boron deficiency and be available for metabolism by these alternate routes. It is thus possible that UDP-glucose pyrophosphorylase plays a key role in the morphogenetic development of organisms with cell walls in general, by its: (a) involvement in sugar nucleotide biosynthesis and, the subsequent synthesis of cell wall material; and (b) peripheral effects on the availability of substrates for other reactions.

**LITERATURE CITED**