Germination of Phaseolus Vulgaris I. Resumption of Axis Growth[1]

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Summary. Growth of the excised axis of *Phaseolus vulgaris* L. (var. White Marrowfat) begins after a 7-hour incubation in buffer or water at 26°. Growth, as measured by axis elongation or fresh weight increase, is linear for at least 8 hours with a resultant fresh weight increase of approximately 65 %. Cell elongation begins 4 or 5 hours prior to cell division and 5 or 6 hours prior to radicle protrusion in the intact seed.

The initiation of axis elongation is apparently dependent on synthesis of RNA and protein. Both actinomycin D and puromycin inhibit the initiation of elongation. Actinomycin D inhibits the incorporation of ATP-8-C^{14} into axis RNA and C^{14}-leucine into protein, while puromycin inhibits the incorporation of C^{14}-leucine into axis protein.

The respiratory rate of the axes increases sharply at about the time of initiation of cell elongation. Dinitrophenol initially increases O_2 uptake by the axes, but at the end of 15 hours the rates of O_2 uptake by control or dinitrophenol-treated axes are approximately the same.

Although seed germination has been studied extensively for many years, relatively few studies have focused on the embryonic axis during the early hours of germination and particularly on the metabolic requirements for initiation of axis growth. Recently, however, several investigators have used excised embryonic axes to study the germination process with interest centered around the requirement for messenger RNA synthesis. In the 2 studies reported, one suggested that the activation or synthesis of messenger RNA is a prerequisite for the germination of peanuts and wheat (13), while the other concluded that messenger RNA synthesis is not required for initial growth of cotton embryos (4).

This paper describes some of the physiological changes occurring in the excised axis of *Phaseolus vulgaris* during the initial 15 hours of incubation, and presents evidence that synthesis of RNA and protein are prerequisites for the initiation of axis elongation.

Methods and Materials

Embryonic axes were excised from the dry seed of *Phaseolus vulgaris* L. (var. White Marrowfat) obtained from the W. Atlee Burpee Company. Both the seeds and the excised axes were stored at 3° for extended periods of time with no noticeable change in subsequent growth.

Most of the growth studies were carried out by incubating 30 to 35 axes on a sheet of filter paper in a petri dish. Three ml of the appropriate incubation media were added and the petri dishes floated in a constant temperature bath. In several experiments axes were incubated in 50 ml Erlenmeyer flasks containing 2.5 ml of solution. The flasks were shaken in a Dubnoff metabolic incubator. The basic control media consisted of 0.01 M potassium phosphate buffer, pH 6.0, to which was added 30 μg per ml streptomycin sulfate. However, incubating the axes in distilled water or at pH values as low as 4.0 did not appear to affect the growth of the axes. At the end of the incubation period, which was generally up to 15 hours, the axes were blotted with tissue paper and weighed to the nearest 0.1 mg. Axis dry weight was obtained by maintaining the axes at 80° until the weight was constant.

Respiration was measured using standard Warburg manometric techniques, except that the axes were placed on filter paper cut-outs on the bottom of the Warburg flasks and 0.25 ml solution added. Three axes were added to each of the 7-ml flasks used for measuring respiration. The respiratory measurements were made on axes which were in the flasks during the entire period of observation.

Axes to be studied for evidence of cell division were immersed in Craf 3 killing solution (17) and dehydrated with an ethanol-tertiary butanol-paraffin oil schedule (20). The paraffin-embedded axes were sectioned and stained with a progressive iron-hematoxylin schedule (17). The tissue was examined microscopically for mitotic figures at 430x magnification.

For RNA analyses, the axes were ground with a mortar and pestle in deionized water at 3°. A modified Smillie and Krotkov procedure was used for the analyses (8). The entire homogenate was made

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0.2 N with respect to perchloric acid, shaken and centrifuged at 1500 × *g* for 15 minutes. The pellets were resuspended and twice washed in 0.2 N perchloric acid and twice in methanol containing 0.02 N formic acid. All centrifugations were carried out at 2° to 4° at 1500 × *g* for 15 minutes. The pellets were then extracted with a 2:2:1 mixture of ethanol-ether-chloroform. RNA was hydrolyzed in 0.5 N KOH for 40 hours at 30° (22). After chilling, perchloric acid was added to a final concentration of 0.3 N and the DNA and protein removed by centrifugation. Absorbance of the RNA samples was measured at 269 mμ with a Beckman DU spectrophotometer. The conversion factor used for converting absorbance to weight of RNA was:

μg RNA/ml of sample read = (A_{269}) × (36).

This conversion factor was obtained by determining the nucleotide composition of the RNA by the method of Zscheile and Murray (22).

When the radioactivity of the RNA samples was to be measured, the pH of the solutions was raised to 5.0 with KOH and the resulting precipitate removed by centrifugation. Aliquots of the supernatant fraction were plated, dried and counted for C^{14}-nucleotide incorporation into RNA in a gas-flow counter, with an efficiency of approximately 20 %.

For determination of C^{14}-leucine incorporation into protein a modification of the procedure reported by Key was used (9). After incubation in C^{14}-leucine, the axes were rinsed and then ground with a mortar and pestle at 2° to 4° in 10 ml solution containing 0.01 M Tris-HCl, pH 7.5, and 0.2 mg/ml C^{12}-leucine. The homogenate was made 5 % in trichloroacetic acid and then centrifuged at 1500 × *g* for 15 minutes. The pellet was washed with 5 % trichloroacetic acid and then suspended in 1 N NaOH. After incubating at 37° for 30 minutes, the suspension was centrifuged at 1500 × *g* for 15 minutes. The pellet did not contain any radioactivity and was discarded. The supernatant was brought to a 5 % trichloroacetic acid concentration and then centrifuged as above. The pellet was washed with 5 % trichloroacetic acid and then dissolved in 2 N NH_4OH. Aliquots from this solution were plated, dried, and counted for radioactivity with a gas-flow counter.

Protein content was estimated by the method of Lowry et al. (12).

ATP-8-C^{14} with a specific activity of 10 mc/mmole was purchased from Schwarz Bio-Research.

Results

Figures 1 and 2 show the course of fresh weight increase by the axes at various temperatures over an incubation period of 15 hours. There is an initial rapid increase of fresh weight due to imbibition, followed by a period of constant fresh weight. The second phase of fresh weight increase, which begins at about the seventh hour of incubation at 26°, is due to axis elongation and is linear over the period of observation. Measurements of the intact axes

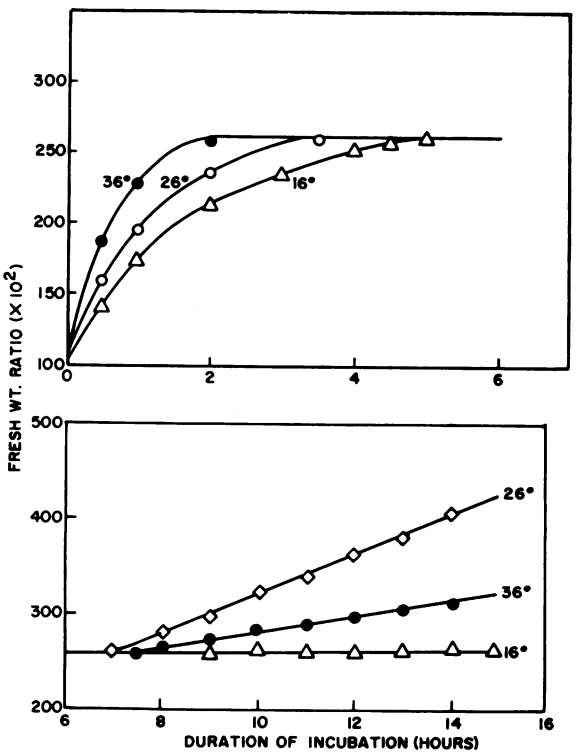

FIG. 1, 2. Fresh weight increase of axes incubated at 16°, 26°, and 36°. Figure 1 (upper). Fresh weight increase during imbibition phase. Thirty axes incubated on filter paper in petri dish. Incubation medium contained 3 ml 0.01 M K phosphate buffer, pH 6.0. Figure 2 (lower). Fresh weight increase during growth phase. Incubation conditions same as those described for figure 1.

Table I. *Effects of Various Treatments on Increase in Axis Fresh Weight between Fourth and Fifteenth Hour of Incubation*

Approximately 95 mg tissue dry weight (30 axes) used per treatment. All solutions contain 0.01 M K phosphate buffer, pH 6.0, and 30 μg/ml streptomycin sulfate. Temperature 26°.

Treatment	Fr wt increase % Control
10 μg/ml actinomycin D*	50
100 μg/ml actinomycin D*	40
10 μg/ml actinomycin D**	40
25 μg/ml actinomycin D**	25
100 μg/ml actinomycin D**	15
10^{-3} M puromycin*	10
10^{-4} M DNP*	0
10^{-5} M IAA*	70
1 mg/ml chloramphenicol*	40

* Axes incubated in 3 ml solution on filter paper in petri dish for entire period.

** Axes incubated in 2.5 ml solution in 50 ml Erlenmeyer flask in Dubnoff shaking incubator for initial 2 hours. At the end of this period, axes transferred to petri dishes and incubated on filter paper with 3 ml solution for remainder of period.

and examination of sectioned tissue indicated that the fresh weight increase was almost entirely due to cell elongation with little or no radial expansion occurring. A correlation coefficient of 0.94 was obtained between the fresh weight increase and axis length. Axis dry weight decreased approximately 15 % over the 15-hour period.

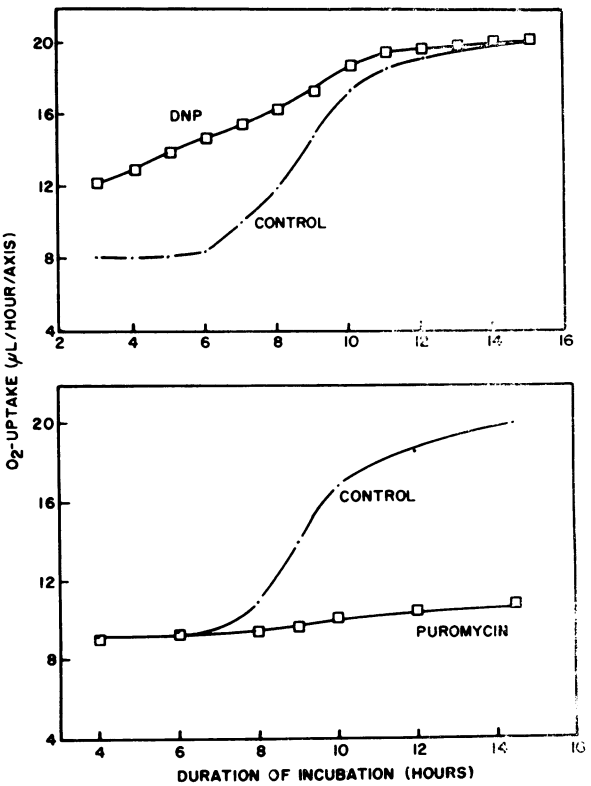

FIG. 3, 4. Axis O_2 uptake during incubation. Figure 3 (upper). Effect of 10^{-4} M DNP on O_2 uptake at 26°. Three axes and 0.25 ml solution in each 7-ml Warburg flask. Axes in DNP for entire period of incubation. All solutions contain 0.01 M K phosphate buffer, pH 6.0, and 30 μg/ml streptomycin sulfate. Figure 4 (lower). Effect of 10^{-3} M puromycin on O_2 uptake. Incubation conditions as described for figure 3.

Table II. *Cytoplasmic Distribution of Protein in Homogenates of Axes Incubated for 4 and 15 Hours*

95 mg tissue dry weight incubated in 3 ml solution containing 0.01 M K phosphate buffer, pH 6.0, and 30 μg/ml streptomycin sulfate at 26°. Tissue homogenized in 15 ml 0.5 M sucrose containing 0.01 M Tris Cl buffer (pH 7.5) and 4×10^{-3} M EDTA.

Subcellular fraction designation*	Length of incubation 4 hrs μg protein/ axis**	15 hrs μg protein/ axis
I	300	321
II	109	119
III	70	90
IV	427	444

* Subcellular fractions: I, 0–1500 × *g* for 15 minutes; II, 1.5–10,000 × *g* for 15 minutes; III, 10–105,000 × *g* for 60 minutes; IV, 105,000 × *g*.

** Initial axis dry weight 3.1 mg; final axis dry weight 2.6 mg.

The imbibitional phase of water uptake is temperature dependent but is not affected by such compounds as actinomycin D, puromycin, 2,4-dinitrophenol (DNP) and IAA which do inhibit the phase of fresh weight increase corresponding to axis growth (table I).

In order to compare growth of the axis in the intact seed with that of the excised axis, whole seeds were germinated at 26° for 24 hours. At the end of this period the radicle was just beginning to protrude through the seed coat. The lengths and fresh weights of the axes corresponded to those of excised axes which had been incubated for 12 to 13 hours.

An examination of the axes for mitotic figures indicated that cell division probably begins at about the eleventh or twelfth hour of incubation. The initial site of cell division was limited to the radicle with no mitotic figures observed in the epicotyl region prior to an incubation period of approximately 24 hours.

The respiratory pattern of the axes is similar to that which has been observed for other seeds and axes (11). The rate of O_2 uptake is constant for several hours and then increases sharply before reaching a plateau (fig 3). DNP initially increases the rate of O_2 uptake but at the end of 15 hours the rates of O_2 uptake for both treated and controls are the same (fig 3).

Puromycin inhibited the increased rate of O_2 uptake that occurs when the axes are incubated in buffer without affecting the initial rate of uptake (fig 4).

Axes excised from seeds which had been germinated intact for 24 hours at 26° showed rates of O_2 uptake comparable with excised axes which had been incubated for 15 hours.

The respiratory quotient was approximately constant over the entire period of incubation with a value of 1.1. Cyanide at a concentration of 10^{-4} M reduced the O_2 uptake of the axes by 90 % both prior to and after the rise in rate.

Tables II and III show that the total protein and RNA content of the axes increases very little over the period of incubation with the result that on a fresh weight basis the concentrations of both decrease over the period of incubation. Only the microsomal fraction showed a significantly higher protein content after a 15-hour period of incubation.

Both RNA and protein synthesis begin prior to the initiation of axis elongation, however, as indicated by incorporation of C^{14}-leucine and ATP-8-C^{14} into protein and RNA, respectively (tables III, IV).

Actinomycin D inhibits the incorporation of ATP-8-C^{14} into axis RNA and C^{14}-leucine into axis protein (tables III, IV). Puromycin inhibits the incorporation of C^{14}-leucine into axis protein (table IV). Both actinomycin D at a concentration of 100 μg/ml and 10^{-3} M puromycin inhibited axis elongation by at least 85 % (table I).

Table III. *Inhibition by Actinomycin D of Incorporation of ATP-8-C^{14} into Axis RNA*

95 mg tissue dry weight incubated in solution containing 0.01 M K phosphate buffer, pH 6.0, 30 μg/ml streptomycin sulfate, 0.25 μc/ml ATP-8-C^{14} (10 μc/μmole). Axes incubated either in petri dishes or in Erlenmeyer flasks. Actinomycin D, at concentration of 100 μg/ml present where indicated throughout incubation period.

Treatment	Interval of incubation with ATP-8-C^{14} (hrs)	μg RNA/axis	cpm in RNA/mg RNA	% Inhibition (cpm in RNA/mg RNA)
Buffer–petri	0–6.5	91	423	...
Actinomycin D–petri	0–6.5	91	275	35
Buffer–Erlenmeyer	0–6.5	91	550	...
Actinomycin D-Erlenmeyer	0–6.5	91	275	50
Buffer–petri	0–15	95	2460	...
Actinomycin D–petri	0–15	91	338	86
Buffer–petri	7–15	95	345	...
Actinomycin D–petri	7–15	91	30	91

Table IV. *Inhibition by Actinomycin D and Puromycin of C^{14}-Leucine Incorporation into Axis Protein*

125 mg tissue dry weight incubated in 2.5 ml solution containing 0.01 M K phosphate buffer, pH 6.0, and 30 μg/ml streptomycin sulfate. Incubation in Erlenmeyer flasks at 26°. Actinomycin D and puromycin added at beginning of incubation. At the end of 4.5 hours, solutions poured off and new solution added containing above plus 0.25 μc C^{14}-leucine (13 μc/μmole). Incubation continued for 2 hours.

Addition	Activity (cpm/g fr wt) In protein	Total uptake
None	25,200	31,200
75 μg/ml Actinomycin D	6700	14,700
10^{-3} M Puromycin	4850	11,700

Discussion

The results show that after a lag of 7 hours axis elongation occurs at a linear rate for at least 8 hours at 26° independent of exogenous nutrients. The resultant fresh weight increase is approximately 65 %.

Comparisons with intact seeds suggest that axis growth precedes radicle protrusion by 6 to 7 hours and that most of the initial growth is due to cell elongation rather than radial growth or cell division. The occurrence of cell expansion prior to cell division during germination has also been reported for barley (2), *Vicia fabia* (21), *and Vigna sesquipedalis* (16).

When the axes were incubated on filter paper for 15 hours with actinomycin D, even at the high concentration of 100 μg/ml, growth was never inhibited more than 60 %. In addition to the failure to effect complete inhibition of the fresh weight increase, this treatment with actinomycin D often caused the axes to assume an almost circular configuration at the end of the incubation period, indicating a differential rate of growth of the axis.

However, when the axes were either first incubated in Erlenmeyer flasks in a 100 μg/ml actinomycin D solution for 2 hours and then transferred to the petri dishes for the remainder of the incubation period, or incubated in Erlenmeyer flasks for the entire period, growth was inhibited by at least 85 %.

The incorporation of ATP-8-C^{14} into RNA was inhibited by approximately 50 % during the initial 6.5 hours of incubation if the axes were shaken in Erlenmeyer flasks, and by 35 % if incubated in petri dishes (table III). Incorporation into RNA was inhibited by approximately 90 % when the axes were incubated for 15 hours in petri dishes (table III). The data suggest the possibility that the failure to inhibit growth completely when the axes were incubated in petri dishes may have been due to incomplete penetration of actinomycin D during the early hours of imbibition.

A recent paper reported that high concentrations of actinomycin D did not inhibit the germination of lettuce seed although subsequent growth of the seedling was inhibited (14). However, the question was raised by the author as to whether the failure of actinomycin D to inhibit germination was due to the lack of a requirement for RNA synthesis or to a permeability barrier. On the other hand, there is also a recent report that cotton embryos incubated for 36 hours in 20 μg/ml actinomycin D showed the same rate of growth as that exhibited by the controls (4). In this case, the authors concluded that there was no requirement for RNA synthesis prior to initiation of embryo growth.

The results obtained with puromycin and chloramphenicol indicate a requirement for protein synthesis in the axes prior to initiation of elongation. Although increases in the activities of various enzymes during germination have been noted many times (11), there is no evidence that the initiation of axis growth is dependent upon their syntheses. Most of the enzymes studied have been of the hydrolytic type and are primarily located in the storage organs. Presumably, they are more directly concerned with the utilization of reserve material than with elongation processes. The activities of 3′ nucleotidase and ribonuclease have been shown to increase in the axes of various seeds during germination (7, 19). It is not known, however, what relationship these enzymes have to initiation of elongation.

The lack of an appreciable net protein synthesis

during elongation has been noted before for excised tissues, including excised embryonic axes (1, 18). It has been pointed out, however, that since plant cells may enlarge their vacuoles without increasing their cytoplasm content, large increases in protein may not be necessary for elongation per se (15). Since some protein synthesis does appear to be necessary for elongation, the synthesis of enzymes directly involved in the elongation process may be sufficient.

It seems quite possible that the protein synthesis required for the initiation of axis growth is not unique to seed germination, but may involve the same compound(s) that are apparently involved generally in plant cell elongation. Key has found that both puromycin and actinomycin D inhibit the continuing elongation of excised soybean hypocotyl tissue (9). With this tissue several hours were required for a high rate of inhibition to occur and it was postulated that the lag was due to the depletion of RNA and protein(s) necessary for continuing cell expansion. More recently, Key and Shannon presented evidence that synthesis of messenger RNA may be a prerequisite for continuing cell elongation in both excised soybean hypocotyl and corn mesocotyl tissue, although synthesis of other types of RNA is also occurring (8).

The effects of DNP and puromycin on axis respiration resemble those that have been observed for potato tuber slices (5). In both tissues DNP increases the initial O_2 uptake, but not the developed respiration. In both tissues puromycin inhibits development of an increased O_2 uptake. In addition, both tissues take up phosphate in much greater quantities after the rate of O_2 uptake has increased (unpublished observation). In the case of the potato slices, it has recently been suggested that the development of respiration depends on the synthesis of specific enzymes which are presumably directly involved in the respiratory increase (3). The results obtained with the *Phaseolus* axes do differ in several respects, however, from those obtained for the potato slices. Most important is the fact that the axes increase in fresh weight by 60% during the respiratory rise while the fresh weight of the potato slices increases only a few percent (6). In addition, the O_2 uptake of the axes is still sensitive to cyanide after the respiratory rise and the activities of several mitochondrial enzymes are still essentially the same (unpublished data).

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