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# $^{31}$P-Nuclear Magnetic Resonance Determination of Phosphate Compartmentation in Leaves of Reproductive Soybeans (*Glycine max* L.) as Affected by Phosphate Nutrition[1]

**Michael J. Lauer, Dale G. Blevins*, and Hanna Sierzputowska-Gracz**

*Department of Agronomy* (M.J.L., D.G.B.) *and Department of Chemistry,* (H.S.-G.) *University of Missouri, Columbia, Missouri 65211*

## ABSTRACT

**Most leaf phosphorus is remobilized to the seed during reproductive development in soybean. We determined, using $^{31}$P-NMR, the effect phosphorus remobilization has on vacuolar inorganic phosphate pool size in soybean (*Glycine max* [L.] Merr.) leaves with respect to phosphorus nutrition and plant development. Phosphate compartmentation between cytoplasmic and vacuolar pools was observed and followed in intact tissue grown hydroponically, at the R2, R4, and R6 growth stages. As phosphorus in the nutrient solution decreased from 0.45 to 0.05 millimolar, the vacuolar phosphate peak became less prominent relative to cytoplasmic phosphate and hexose monophosphate peaks. At a nutrient phosphate concentration of 0.05 millimolar, the vacuolar phosphate peak was not detectable. At higher levels of nutrient phosphate, as plants progressed from the R2 to the R6 growth stage, the vacuolar phosphate peak was the first to disappear, suggesting that storage phosphate was remobilized to a greater extent than metabolic phosphate. Under suboptimal phosphate nutrition (≤ 0.20 millimolar), the hexose monophosphate and cytoplasmic phosphate peaks declined earlier in reproductive development than when phosphate was present in optimal amounts. Under low phosphate concentrations (0.05 millimolar) cytoplasmic phosphate was greatly reduced. Carbon metabolism was coincidently disrupted under low phosphate nutrition as shown by the appearance of large, prominent starch grains in the leaves. Cytoplasmic phosphate, and leaf carbon metabolism dependent on it, are buffered by vacuolar phosphate until late stages of reproductive growth.**

In mature leaf tissue, much of the leaf phosphorus is believed to be stored in the vacuole (1–3). One way to examine the distribution of Pi between the vacuole and cytoplasm (nonvacuolar parts of the cell) is with $^{31}$P-NMR (22, 23, 25). Recent applications of $^{31}$P-NMR to living plant tissues have helped illuminate the relationship between Pc[2] and Pv pools (13, 14, 23, 24). $^{31}$P-NMR allows one to differentiate between the Pc and Pv pools due to the different pH environments of the Pi molecules (23, 24). Typically, the vacuole has an approximate pH of 5.5 and the cytoplasm has an approximate pH of 7.0, producing a spectral shift for Pi of about 1.5 ppm. The pH gradient permits one to differentiate between Pi in the cytoplasmic and vacuolar pools. In addition, the area under the NMR peak is proportional to the amount of phosphorus in a particular environment (23, 24), allowing one to make quantitative comparisons of compartment Pi content.

It has been estimated that 90% of leaf Pi can be sequestered in the vacuole (2, 3). Vacuolar phosphate is slowly released into the cytoplasm (29) and is therefore not readily available for metabolism. NMR is capable of providing new information regarding carbon metabolism, Pi compartmentation, and phosphorus remobilization *in vivo*. Many $^{31}$P-NMR studies have focused on pH changes in the tissue (13, 15, 23–25), but others have looked at Pi compartmentation relating it to metabolism (7, 15–17, 20, 26, 28).

Rebeille *et al.* (20), were able to quantify Pv and Pc in *Acer pseudoplatanus* cell suspension cultures using $^{31}$P-NMR. The Pi content of the cells was varied by deletion or addition of Pi to the culture medium, and the time course of Pv and Pc adjustments were followed with $^{31}$P-NMR. When Pi was withheld, the Pv peak decreased while the Pc peak stayed relatively constant. When Pi was provided to Pi starved cells, the Pv peak increased while the Pc peak again stayed relatively constant (20), suggesting that, in this system, Pc is maintained at a relatively constant level.

The ability of $^{31}$P-NMR to distinguish Pc and Pv in leaf tissue was demonstrated by Waterton *et al.* (28). More recently, Foyer and Spencer (7) used $^{31}$P-NMR to quantify Pi compartmentation in leaf tissue. Barley grown for 34 d in the absence of added phosphate had no detectable Pv (7). Additionally, as Pi was withheld from barley which had been grown in nutrient solution containing 1.0 mM Pi, the Pv peak decreased, while the Pc peak was less affected (7).

The importance of phosphorus in reproductive development is illustrated by its remobilization pattern (10, 11). A greater percentage of the total phosphorus taken up is remobilized to the seed than any other nutrient element in the plant, including N (4). During soybean seed filling enormous amounts of phosphorus are remobilized and transferred to

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[2] Abbreviations: Pc, cytoplasmic inorganic phosphate; Pv, vacuolar inorganic phosphate; HMP, hexose monophosphate.

the seed (10, 11), even though Pi is still needed to carry out metabolic functions. Approximately half of the phosphorus in the seed is translocated from other plant parts, with the remainder taken up from the soil during seed development (10). Phosphorus remobilization may cause increased phosphorus stress in leaves. Remobilization effects on Pc and Pv have not yet been reported. Our objective was to determine the buffering effect of vacuolar Pi on maintaining cytosolic Pi during the period of phosphorus remobilization as pods fill.

## MATERIALS AND METHODS

### Plant Culture

Soybeans (*Glycine max* [L.] Merr. cv Williams 82) were grown in the greenhouse in hydroponic culture. The seeds were imbibed for 24 h with distilled and deionized water, and then planted on May 27, 1987 in perlite at 2.5 cm and inoculated with a commercial preparation of *Rhizobium japonicum* (Research Inoculants, St. Joseph, MO). Plants were thinned at the unifoliolate stage to 3 plants per pot. Eight replications were established to obtain data for dry weight and phosphorus partitioning (data not shown) but all replications were pooled to obtain sufficient leaf material of uniform age for the NMR experiments. The soybeans were grown on a standard nutrient solution containing: 4.0 mM $CaCl_2$, 1.25 mM $K_2SO_4$, 0.5 mM $MgSO_4$, 26.0 $\mu$M Fe citrate, 2.3 $\mu$M $H_3BO_3$, 0.9 $\mu$M $MnSO_4$, 0.6 $\mu$M $ZnSO_4$, 0.15 $\mu$M $CuSO_4$, 0.10 $\mu$M $NaMoO_4$, 0.01 $\mu$M $CoCl_2$, 0.11 $\mu$M $NiCl_2$, and varying levels of phosphate. The control treatment (treatment A) contained 0.45 mM $K_2HPO_4$, an amount previously determined to be optimal for Williams 82 soybeans under summer greenhouse conditions (9). The other treatments contained either 0.20 mM $K_2HPO_4$ (treatment B), 0.10 mM $K_2HPO_4$ (treatment C), or 0.05 mM $K_2HPO_4$ (treatment D), with the overall potassium concentration being maintained by the appropriate addition of 0.5 M $K_2SO_4$.

### $^{31}$P-NMR

The youngest, fully expanded leaves from each treatment were harvested when plants were in the R2 (full flower), R4 (full pod), and R6 (full seed) growth stages (6). Leaves were deveined, sliced into 3 mm pieces and infiltrated with NMR buffer (300 mM Sorbitol in 50 mM Hepes [pH 7.5]) (7). Infiltration was achieved by spinning the tissue in buffer while repeatedly applying and removing a vacuum. $^{31}$P-NMR spectra of noninfiltrated leaves yield broad peaks, due to the large intracellular air spaces within the leaf (28). Infiltration with buffer increased resolution of the spectra by reducing the peak width due to air spaces. Approximately 6 g fresh weight was packed into 20 mm NMR tubes, and about 3 g fresh weight was in the limit of the observation coil of the spectrometer. A perfusion system similar to that used by Roby *et al.* (26) provided aerated buffer to the tissue at a rate of 12 ml per min during spectra collection. Observation times were limited in some cases by the tissue going anaerobic, in which case, the Pc and Pv peaks would merge as the cytoplasm became more acidic (22, 23, 25). In all cases, a compromise was reached between the amount of tissue packed in the NMR tube and ability to perfuse aerated buffer past the tissue.

NMR spectra were obtained with a Nicolet 300-MHz ($^1$H) Fourier transform spectrometer with an Oxford Instruments wide bore magnet and Nicolet 1280 computer. The instrument was equipped with a Nicolet 20 mm single frequency $^{31}$P probe (121.46 MHz). Phosphorus spectra were recorded at the frequency of 121.46 MHz in a window of ±5000 Hz, with a 75° pulse width of 30 $\mu$s and a repetition time of 1.5 s. We determined that these conditions permitted 70% relaxation of Pi between pulses in our system. All spectra were adjusted to reflect this correction term. Rebeille *et al.* (20) reported that a 1.36 s repetition time allowed complete relaxation of Pv and Pc under their experimental conditions, and Lee and Ratcliffe (14) quantified intracellular Pi using a repetition time of 0.26 s. Methylene diphosphonic acid (50 mM in 30 mM Tris [pH 8.9]) in a sealed capillary was included in the NMR tube during spectra collection as an external standard (23). As peak area is directly related to Pi concentration in a particular chemical environment (23, 24), a standard curve was generated using phosphorus concentrations ranging from 0.05 to 10.0 mM $KH_2PO_4$ in 50 mM KCl. The NMR conditions used to obtain the standard curve were similar to those used on the leaf tissue; however, we defined the delay time for each standard concentration based on the Ernst equation. The lower limit of detection in our system was 0.1 mM phosphorus in 0.1 h. Spectra were plotted with a line broadening of 10 Hz.

### Electron Microscopy

Tissue from the youngest fully expanded leaves of the 0.45 and 0.05 mM Pi treatments at the R2 growth stage was fixed in 2.5% glutaraldehyde (pH 7.0) in a 0.1 M phosphate buffer at 4°C for 4 h. Samples were postfixed in 1% osmium tetraoxide in 0.1 M phosphate buffer at 4°C for 4 h. The samples were dehydrated in a graded series of 20 to 100% acetone, then embedded in Epon. Ultrathin sections were made, and the tissue was stained, first with uranylacetate, and then with lead citrate. Photographs were taken with a JEOL model 100B by Dr. Jerry White of the University of Missouri College of Agriculture Electron Microscope facility.

## RESULTS AND DISCUSSION

Phosphate compartmentation in intact soybean leaves was investigated with $^{31}$P-NMR. The experiment reported here is a follow-up on a series of preliminary experiments which yielded similar results. The spectra shown in Figures 1, 2, and 3 contain peaks at spectral shifts of −13.0, −14.7, and −16.3 ppm, referenced to methylene diphosphonic acid at pH 8.9. Based on published spectra (23) these peaks have been assigned to HMP at −13.0 ppm, Pc at −14.7 ppm, and Pv at −16.3 ppm. The broad peak at −27 to −28 ppm corresponds to the $\alpha$-nucleotide triphosphate peak (23). Although we infiltrated the leaf tissue with buffer to reduce line broadening due to intracellular air spaces, the peaks were still rather broad, due in part to the heterogeneity of cell types in the leaves (28). The area under each of the HMP, Pc, and Pv peaks was measured and compared to the peak area generated

by Pi standards to estimate the Pi content of these compartments. Results are tabulated in Table I as compartment Pi content, but due to the large signal to noise, and the broad peaks, these figures should be taken as relative comparisons rather than absolute amounts. The number of scans accumulated to obtain each spectrum is noted in the figure legends. Figures have been normalized for the number of scans accumulated for each spectrum and for instrument scaling.

The spectrum in Figure 1A is from intact leaves of soybeans at the R2 growth stage, grown in nutrient solution containing 0.45 mM Pi (treatment A). Comparison of this spectrum to that from leaves grown in 0.2 mM Pi (treatment B) and 0.1 mM Pi (treatment C) revealed a decline in all Pi peaks, and a progressive decline in the Pv peak (Fig. 1, B and C), until the Pv peak was less than 0.10 mM (Fig. 1C; Table I). The HMP and Pc peaks also showed a progressive decline as the Pi in the nutrient solution decreased (Fig. 1, A, B, and C), until the Pc peak was 0.23 mM Pi in the tissue grown at 0.05 mM Pi (treatment D). The Pc peak of treatment D is detectable as a shoulder on the larger HMP peak in spectrum 1D which is enlarged 11 times relative to treatment A (Fig. 1A). These data suggest that soybean leaves allocate Pi to the Pc pool at the expense of the Pv pool under decreasing Pi availability. Rebeille *et al.* (20) reported decreased Pv as Pi was withheld from *Acer pseudoplatanus* cell cultures. A decrease in Pv was also reported for barley grown in the absence of added Pi (7). Further, in our study, as Pi deficiency became more extreme, the Pc pool became increasingly depleted but the HMP pool was maintained to a greater extent.

Indeterminate soybeans in the R2 growth stage are engaged in rapid vegetative growth while entering reproductive growth. The demand for structural and metabolic phosphorus may therefore be quite high at this point in plant development. Demand for structural and metabolic phosphorus may be expected to increase by the R4 growth stage because the indeterminate soybean plant at this stage is engaged in both vegetative growth and rapid reproductive growth (21). The $^{31}$P-NMR spectra from R4 soybean leaves are shown in Figure 2. While the Pv content had increased in treatment A, from 8.01 to 13.56 mM Pi, it had decreased greatly, from 3.50 to 0.51 mM Pi in treatment B and was less than 0.05 and 0.025 mM in treatments C and D, respectively. The HMP and Pc pools were maintained in treatment A but decreased in treatments B and C (Table I). Treatment D already had very low levels of phosphorus in the HMP and Pc pools and exhibited little change as these plants progressed from R2 to R4. In treatment A, the supply of Pi was great enough to maintain the HMP and Pc pools while permitting further accumulation of Pv in spite of the concurrent demands of vegetative and reproductive growth.

The data in these spectra further suggest that the Pv pool is filled only after the demand for phosphorus in the HMP and Pc pools is met, for, as in Figure 1, the Pv pool became progressively smaller as the available Pi decreased (Fig. 2, A, B, and C). Although the Pc and HMP peaks were reduced in treatments B and C, they were less affected than Pv. Additionally, the plant maintained a detectable Pc peak at this point in plant development even under the lowest Pi level tested (Fig. 2D). A comparison of the HMP and Pc peaks across treatments in Figure 2, shows that the plant is main-

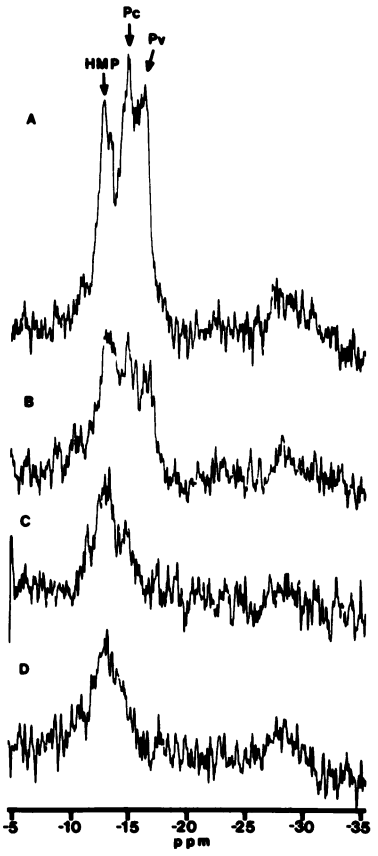


**Figure 1.** $^{31}$P-NMR spectra of soybean leaves at the R2 growth stage, grown on 0.45 (A), 0.2 (B), 0.1 (C), or 0.05 (D) mM Pi. Spectra are referenced to methylene diphosphonic acid as an external standard. Peak assignments were made in reference to the literature (23) as: HMP at −13.0 ppm, Pc at −14.7 ppm, and Pv at −16.3 ppm. The vertical expansion of the spectra, normalized for number of scans and instrument scaling, are: 1×, A; 1×, B; 3×, C; and 11×, D. Spectra were accumulated for: 1004 scans, A; 1008 scans, B; 2612 scans, C; and 10692 scans, D.

**Table I.** *Estimated Phosphate Contents of Soybean Leaf Compartments as Affected by Phosphate Nutrition and Reproductive Growth, Based upon $^{31}$P-NMR Spectra*

| Treatment | Growth Stage | Phosphate Peaks | | |
|---|---|---|---|---|
| | | HMP | Pc | Pv |
| *mM Pi* | | | *mM* | |
| 0.45 | R2 | 7.65 | 8.32 | 8.01 |
| 0.20 | | 5.75 | 3.56 | 3.50 |
| 0.10 | | 2.11 | 0.87 | <0.1 |
| 0.05 | | 0.54 | 0.23 | <0.05 |
| 0.45 | R4 | 8.98 | 7.59 | 13.56 |
| 0.20 | | 1.24 | 0.93 | 0.51 |
| 0.10 | | 0.81 | 0.69 | <0.05 |
| 0.05 | | 0.42 | 0.23 | <0.025 |
| 0.45 | R6 | 4.10 | 5.63 | 7.65 |
| 0.20 | | 0.78 | 0.72 | <0.1 |
| 0.10 | | 0.39 | 0.21 | <0.05 |
| 0.05 | | <0.01 | <0.01 | <0.01 |

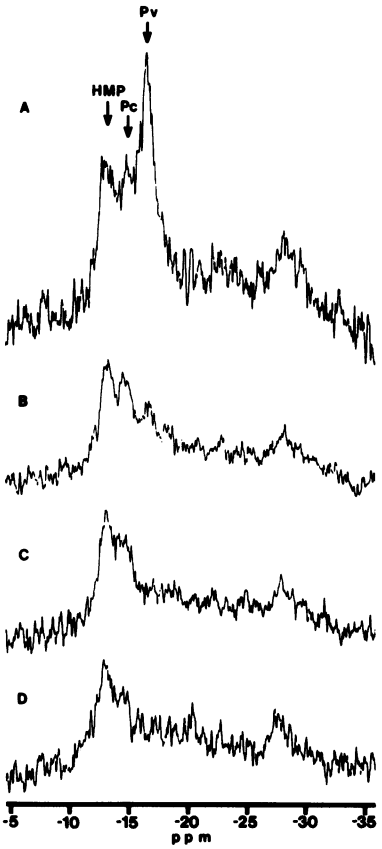


**Figure 2.** $^{31}$P-NMR spectra of soybean leaves from plants at the R4 growth stage. Peak assignments were made as in Figure 1. The vertical expansion of the normalized spectra are: 0.5×, A; 3×, B; 5×, C; and 12×, D. Spectra were accumulated for: 524 scans, A; 2792 scans, B; 5000 scans, c; and 11504 scans, D.

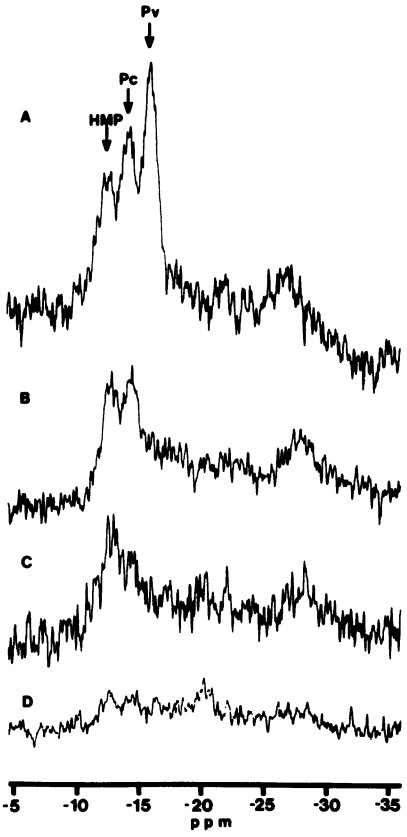


**Figure 3.** $^{31}$P-NMR spectra of soybean leaves at the R6 growth stage. Peak assignments were made as in Figure 1. The vertical expansion of the normalized spectra are: 1×, A; 5×, B; 9×, C; and 26×, D. Spectra were accumulated for: 1032 scans, A; 2704 scans, B; 4544 scans, C; and 27192 scans, D.

taining both the HMP and Pc pools across the range of Pi nutrition. Indeed, for treatments A and B, the ratio of the Pi content in the HMP and Pc peaks is roughly equivalent, even though the signal from treatment A is about 10 times stronger than that of treatment B, indicating the importance of the Pc pool. Inorganic phosphate is important in plant metabolism, for Pi has been shown to be necessary for carbon export from the chloroplast (12). Inorganic phosphate is an activator of ADP-glucose pyrophosphorylase (18), an inhibitor of sucrose phosphate synthase (5), and a regulator of fructose-2,6-bisphosphate levels (19). Maintenance of a constant HMP/Pc ratio suggests the importance of the Pc pool for plant metabolism *in vivo*.

The R6 growth stage of soybean is characterized by rapidly increasing seed weight. Vegetative growth has ceased but the remobilization of phosphorus from vegetative tissue to the seeds has reached its maximum rate (10, 11, 21). Inorganic phosphate mobilization from the Pv pool is greatly affected by Pi nutrition. The Pv pool was < 0.05 mM Pi in treatment C, and <0.01 mM Pi in treatment D; both were below the limit for detection in the amount of time they were in the magnet (Fig. 3). While one can see the Pv pool disappear from treatment B leaves by comparing Figures 1B, 2B, and 3B, in treatment A, progression from the R4 to R6 growth stage resulted in a large decrease in both the Pv and HMP peaks (Table I). Perhaps the large decrease in HMP is a reflection of decreased carbon fixation in the aging soybean leaf. Earlier work has established 0.45 mM P as the optimal level of Pi for soybean culture under our conditions (9). These data suggest that this optimal level of Pi allows the plant to maintain all leaf Pi pools for a longer time during reproductive development.

Our hypothesis was that during rapid seed development, the Pv pool would be remobilized before the HMP and Pc pools. Results from the 0.2 mM Pi treatment (Table I) best demonstrate the preferential mobilization of Pi from the vacuole to maintain the Pc concentration for metabolism. In this treatment, as the soybean progressed through growth stages R2, R4, and R6, Pc was maintained at 3.56, 0.93, and 0.72 mM P while the Pv dropped to 3.50, 0.51, and less than 0.10 mM P, respectively. Considering that the vacuole makes up 75 to 95% of the leaf volume (27, 28), it appears that Pv was preferentially mobilized during reproductive growth and that the HMP and Pc pools were maintained longer during seed development. That the majority of leaf phosphorus is remobilized during soybean seed development has been established (10, 11). This report demonstrates the preferential

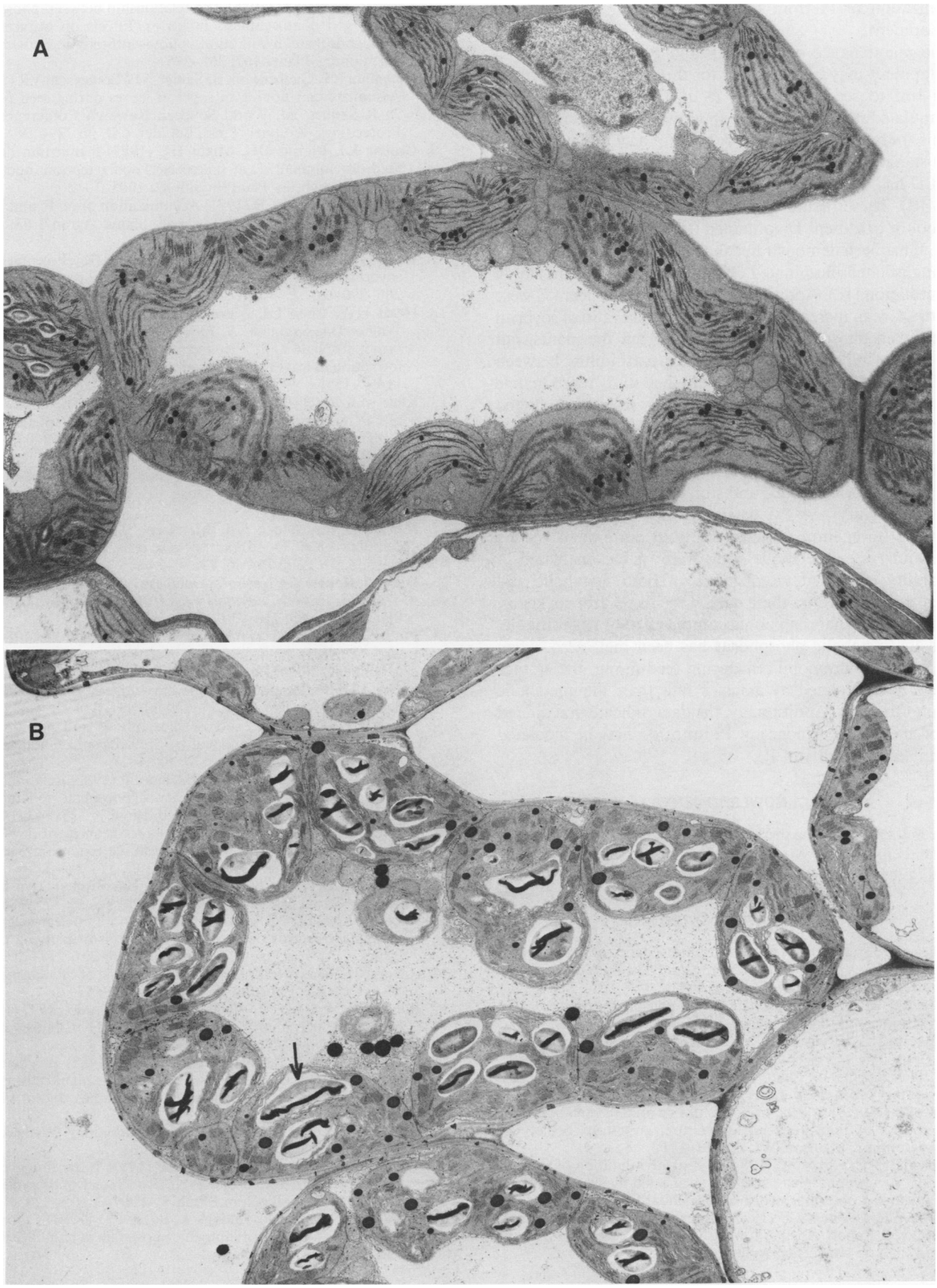


**Figure 4.** Electron micrograph (magnification = ×4600) of the most recently fully expanded leaf of soybeans at the R2 growth stage, grown on 0.45 mM P (A) or 0.05 mM P (B). The arrow in 4B indicates a prominent starch grain.

mobilization of Pi from the vacuole during soybean seed development.

The maintenance of the HMP and Pc pools during seed development may be important for the movement of carbon from leaf to seed. Giaquinta *et al.* (8) found that starch accumulated in leaves of soybean grown on low Pi nutrition. In this study, while treatment A had not accumulated significant starch when harvested at 11:00 h CDT (Fig. 4A), treatment D had accumulated massive starch grains in the leaves (Fig. 4B). These tissues were harvested at the R2 stage. The Pc pool in treatment D contained 0.23 mM Pi at this growth stage. It has been demonstrated that a lack of Pi in the medium bathing isolated illuminated chloroplasts can result in starch accumulation (12). Additionally, Foyer and Spencer (7) were able to show an increase in the starch/sucrose ratio of soybean leaves when phosphorus was withheld from the plants, but they did not report on the phosphorus partitioning between HMP, Pc, and Pv in soybean, nor did they study reproductive plants. Insufficient Pi nutrition apparently resulted in altered carbon metabolism in this study as demonstrated by the large starch grains in the low Pi treatment.

The soybean plant appears to selectively distribute leaf phosphorus into metabolic and storage pools. At lower levels of Pi availability, the Pv, or storage phosphorus pool, was below the lower limit of detectability in our system yet the HMP and Pc, or metabolic pools were visible. Increased Pi availability permitted visualization of both metabolic and storage pools. Based on these data, it seems that for soybeans, the storage phosphorus pool accumulates only when the demand for metabolic phosphorus has been met. Also, when phosphorus was remobilized during seed-filling, the storage Pi pool was depleted at a faster rate than the metabolic phosphorus pools. Additionally, the data indicate that altered carbon metabolism under low Pi nutrition may be mediated by limited availability of Pc.

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