Phosphorus Nutrition Influence on Starch and Sucrose Accumulation, and Activities of ADP-Glucose Pyrophosphorylase and Sucrose-Phosphate Synthase during the Grain Filling Period in Soybean

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ABSTRACT
Several lines of evidence indicate that the partitioning of photosynthesize between starch and sucrose is influenced by the relative concentrations of inorganic phosphate (Pi) in the cytosol and chloroplast. Two greenhouse experiments were conducted to determine the influence of long-term differences in soil P levels, ranging from deficient to supraoptimum, on leaf starch and sucrose concentrations, and activities of adenosine diphosphate glucose (ADPG) pyrophosphorylase and sucrose-phosphate synthase (SPS) during the grain filling period in soybean (Glycine max [L.] Merr.). It was hypothesized that, compared with optimum P nutrition, leaf starch and sucrose concentrations would be increased and decreased, respectively, for P deficiency and visa versa for supraoptimum P nutrition. Relative to the optimum soil P level, leaf Pi concentration was not altered by P deficiency but was increased two- to fourfold for the supraoptimum soil P treatment. The concentrations of leaf starch and sucrose were not markedly affected by any of the P fertility treatments and were not closely related to the activities of ADPG pyrophosphorylase and SPS. P deficiency resulted in increased activity of both enzymes in one of the experiments. The results indicated that long-term soil P treatments, that caused either large decreases in plant growth (P deficiency) or large increases in leaf Pi concentration (supraoptimum P), did not markedly alter starch and sucrose metabolism. Furthermore, it can be inferred that the method of plant culture and/or imposition of the P treatments is a critical factor in interpreting results of P nutrition studies.

The regulatory effect of Pi on the partitioning of photosynthesize between sucrose and starch has been extensively studied (12, 13, 18, 28, 29, 31–33). The exchange of cytosolic Pi with triose phosphates from the chloroplast is thought to be a key component in the regulation of starch and sucrose synthesis (19). Therefore, treatments, such as mannose or glycerol feeding, that sequester Pi external to the chloroplast lead to enhanced flow of photosynthesize into starch (2, 23). Extremely low P fertility levels or removal of P from the nutrient medium at various times during plant development lead to enhanced starch accumulation by leaves (13, 14, 16, 21, 25). In general, treatments that alter the relative concentrations of cytosolic and chloroplastic Pi would be expected to alter starch and sucrose synthesis and accumulation. In some cases, alterations in starch and sucrose accumulation have been associated with corresponding changes in enzymes of starch and sucrose metabolism (13). It has been proposed that P deficiency during the seed filling period may decrease yield in soybean by decreasing the flow of carbon into sucrose that could be used for seed development (15).

There are a few reports that indicate that P stress does not necessarily result in enhanced starch and/or decreased sucrose accumulation (8, 11, 29). In a previous study of vegetative development in tobacco (Nicotiana tabacum L.), it was demonstrated that leaf sucrose and starch concentrations were not markedly affected in plants grown at soil P levels ranging from severely deficient to supraoptimum (8). It was proposed that the variable effects of soil P level on starch and sucrose synthesis and accumulation may be due to experimental procedures such as the use of isolated chloroplasts versus leaves, the timing of application of P nutrition treatments, cultivars used, or the stage of development at which plants were studied.

In an agricultural context, P stress or P excess is likely to begin at seed germination and to develop gradually over time. By altering the P fertility using a soil-based culture system, it is possible to simulate P deficiency or supraoptimum P fertility as they would occur under field conditions. In this culture system, P levels are established at the time of planting with no subsequent addition or removal of P. We have reported results from this type of system for tobacco during vegetative development (8) and soybean during reproductive development (5, 6). In both cases, P deficiency could not be readily detected by comparing leaf P concentrations of plants grown at deficient and optimum soil P levels. Plant growth adapted such that leaf P concentration was maintained at similar levels for plants that differed widely in biomass production and/or seed yield until P fertility exceeded the level needed for maximum growth. At supraoptimum soil P levels, leaf P concentration was markedly increased (5, 6, 8).

We previously reported that seed growth and leaf senescence in soybean were not closely associated with leaf P

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concentration or remobilization of leaf P (5, 6). Here we report the effect of long-term differences in soil P levels on accumulation of leaf Pi, starch, and sucrose, and on the activities of SPS2 and ADPG pyrophosphorylase, two regulatory enzymes in the synthesis of sucrose and starch, respectively.

**MATERIALS AND METHODS**

**Plant Culture**

Plant culture methods for these experiments have been described in detail in previous reports (5, 6). Briefly, two greenhouse experiments were conducted using the soybean (Glycine max [L.] Merr.) cultivar McCall. Planting dates were January 16, 1990 (experiment 1) and March 7, 1990 (experiment 2). Plants were grown in 20 by 20 cm pots, two plants per pot, that contained soil that was very low in available P. Plants were not nodulated due to the high level of N fertility.

Soil P treatments were established by the preplant addition of Ca(H2PO4)2 equivalent to 134, 538, and 2150 kg P ha⁻¹, denoted as P1, P2, and P3, respectively, for experiment 1. For experiment 2, the P1 and P2 treatments were the same as for experiment 1, but the P3 treatment was decreased to 1612 kg P ha⁻¹. Soil test results for extractable P at the beginning of the experiments were previously reported (5). As a reference, the P2 treatment resulted in soil P tests that are considered to be very high.

**Sampling**

Trifoliate leaves from the third or fourth node from the apex of the plant were sampled at growth stages R5 (beginning seed), R6 (full seed), R7 (physiological maturity), and approximately R8 (full maturity) (10). The R8 sampling actually occurred prior to R8 so that attached leaves could be sampled. Leaf area was determined and leaf discs were sampled and frozen at -80°C prior to enzyme assay. The rest of the leaf was frozen and lyophilized prior to dry weight determination. The tissue was then powdered with liquid N prior to chemical analysis.

The experimental design was a randomized complete block design with four replications. At each sampling time, eight leaves, two from each of four pots, were sampled for each P treatment. The data were statistically analyzed by analysis of variance and, when a significant F test was obtained, the LSD (α = 0.05) test was used to compare P treatments within and over sampling times.

Pi

Pi was extracted from 20 mg of dry, ground tissue by shaking for 2 h at 35°C in 50 mM Hepes-NaOH, pH 7.0, and 1.7% (w/v) SDS. Aliquots of the filtered extract were analyzed for Pi by the method of Chifflet et al. (3).

Nonstructural Carbohydrates

Total extractable sugars (reducing sugars, hydrolyzed sucrose, and other hydrolyzed nonreducing sugars) and total extractable nonstructural carbohydrates were colorimetrically determined following extraction of 50 mg subsamples of dry, ground tissue as previously described (17). Starch, expressed as glucose equivalents, was calculated as the difference between total extractable nonstructural carbohydrates and total sugars multiplied by 0.9.

Sucrose was extracted from 60 mg subsamples of dry, ground tissue and directly determined by the enzymatic method of Birnberg and Brenner (1). The tissue was extracted in 10 mM K2PO4 buffer, pH 7.0, and filtered prior to analysis.

ADPG Pyrophosphorylase Activity

ADPG pyrophosphorylase was extracted by homogenizing 113 mm² of leaf area in 0.75 mL of ice-cold buffer containing 50 mM Hepes-NaOH, pH 8.0, 2 mM EDTA, 2 mM MgCl₂, 2 mM glutathione, 2 mM DTT, 10 g L⁻¹ casein, 10 g L⁻¹ PVP, 0.5 mM L⁻¹ Triton X-100, 1 mM PMSF, and 20 μM leupeptin. Extracts were centrifuged for 30 s at 13,000g and 100 μL aliquots of the crude extract were immediately assayed for enzyme activity. The assay was a modification of the spectrophotometric method of Sowokinos (30). Assays were conducted at 30°C in a 1 mL volume containing 40 mM Hepes-NaOH, pH 8.0, 10 mM KF, 5 mM MgCl₂, 0.5 mM L⁻¹ Triton X-100, 0.31 mM NADP, 250 μg BSA, 200 μg glucose-1,6-bisphosphate, 1 mM ADPG, 2 mM 3-phosphoglyceric acid, 0.6 mM Na₃P₂O₆, 1.5 units phosphoglucomutase and 5 units glucose-6-phosphate dehydrogenase. Assays were initiated with Na₃P₂O₆ and values were corrected for endogenous NADP reduction in the absence of pyrophosphate. The correction for pyrophosphate-independent reduction of NADP was critical because this background varied for the P fertility treatments. The background was much higher for the low P treatment (data not shown). Because nondesalted crude extracts were used for the assays, the level of Pi in the extracts was determined by the method of Chifflet et al. (3). For the highest P fertility treatment (P3 in experiment 1) the Pi concentration in the assay was less than 70 μM. Leaf extracts contain very high activities of UDPG pyrophosphorylase that could potentially cause erroneous ADPG pyrophosphorylase activity measurements if ADPG is utilized as a substrate for UDPG pyrophosphorylase. However, it was determined that ADPG was not utilized as a substrate by isolated UDPG pyrophosphorylase (M.E. Salvucci, unpublished data).

SPS Activity

SPS activity was extracted by homogenizing 226 mm² of leaf area in 0.75 mL of ice-cold buffer containing 50 mM Hepes-NaOH, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 2.5 mM DTT, 10 g L⁻¹ casein, 10 g L⁻¹ PVP, 0.5 mM L⁻¹ Triton X-100, 1 mM PMSF, and 20 μM leupeptin. After centrifugation at 13,000g for 30 s, 0.3 mL of the supernatant was desalted at 4°C on an 8 × 40 mm column of Sephadex G50-300 (Pharmacia, Inc., Piscataway, NJ) that was previously equilibrated with extraction buffer. SPS was assayed for 15 min at 30°C by substrate-dependent formation of sucrose. Assays

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2 Abbreviations: SPS, sucrose-phosphate synthase; ADPG, adenosine diphosphate glucose.
were initiated by addition of extract (45 µL) to a reaction mix (150 µL final volume) containing 50 mM Hepes-NaOH, pH 7.4, 15 mM MgCl₂, 1 mM EDTA, 7.5 mM fructose-6-phosphate, 7.5 mM uridine 5'-diphosphate glucose, and 37.5 mM glucose-6-phosphate. Blanks lacking fructose-6-phosphate and glucose-6-phosphate were included for each sample. Assays were terminated by addition of 150 µL of 1 M NaOH. The sucrose formed in the reaction was determined colorimetrically as described by Doehlert and Huber (9).

RESULTS AND DISCUSSION

The effects of the soil P treatments used in these experiments on plant growth and development have been reported (5, 6). Briefly, plant ontogeny was not altered by the soil P treatments. The P1 treatment, however, resulted in plants that produced approximately 30% of the biomass and seed yield of the P2 and P3 treatments. Even though experiment 2 had a much more favorable light environment, relative treatment responses were similar for both experiments.

Treatment differences in leaf Pi concentration (Fig. 1) both within and across sampling times were, on a relative basis, similar to results for leaf total P (5, 6). For both experiments, the P3 treatment resulted in large increases in leaf Pi concentration, and over time the Pi concentration either increased or remained relatively constant (Fig. 1). The leaf Pi concentration was similar for the P1 and P2 treatments for both experiments. These results indicated that the plants adapted to the soil P treatments such that both Pi (Fig. 1) and total P (6) concentrations in leaves were maintained at similar levels at deficient and optimum (i.e., just enough P to attain maximum growth) soil P levels.

It was not known if the soil P treatments altered the Pi concentration within the cytosol or chloroplast. Previous research indicates that the cytosolic Pi concentration is maintained relatively constant by uptake or release of Pi from vacuoles (12, 22, 26, 27, 35). Other studies, however, indicate that differences in P nutrition do alter the cytosolic Pi concentration (21, 34). Whether or not the Pi concentration in various cellular compartments was altered, the soil P treatments established in these experiments are representative of how plants grown under standard agronomic conditions may experience differences in P nutrition.

Figure 1. Pi concentration of leaves sampled from soybean plants that were grown at three different soil P levels. The results of two separate experiments are reported. Soil P levels were 134, 538, and 2150 kg P ha⁻¹ for P1, P2, and P3, respectively, for experiment 1. For experiment 2, the P3 treatment was 1612 kg P ha⁻¹. F LSD (α = 0.05) values for individual sampling times were indicated on the figure when a significant F value was obtained in the analysis of variance.

Because leaf total P (5, 6) and Pi (Fig. 1) concentrations were similar for the P1 and P2 treatments, it was not surprising that leaf starch concentration was similar for these treatments (Fig. 2). Additionally, the excess leaf Pi caused by the P3 treatment did not influence the leaf starch concentration relative to the other treatments (Fig. 2). Other studies with soybeand using hydroponic culture with either seedlings (13) or plants at the full bloom growth stage (21) indicated that deficient P nutrition caused increased leaf starch accumulation. Freenen et al. (13) also reported that root starch was increased and leaf sucrose was decreased by deficient P nutrition. In our study, root starch concentration was very low (approximately 8 mg/g dry weight) and was not influenced by the soil P level (data not shown). It has also been reported that deficient P nutrition resulted in starch differences, predominantly at the end of a dark cycle (16, 20). However, results from our study of vegetative growth of tobacco (8), in addition to the present study of reproductive development in soybean, indicated that long-term differences in soil and/or plant P levels did not significantly alter leaf starch concentration.

There were no consistent treatment differences in leaf sucrose concentration for either experiment (Fig. 3). Therefore, similar to starch, long-term differences in soil P levels did not significantly affect leaf sucrose concentration even though the treatments caused large differences in plant growth and/or leaf Pi concentration. It is possible that instantaneous partitioning of photosynthesis into starch and sucrose was affected by the soil P treatments. However, in view of the lack of effect of the soil P treatments on plant ontogeny, photosynthesis, and leaf senescence (5, 6), it appears more likely that plants grown at deficient or supraoptimum soil P levels simply
adjusted their growth rate or Pi compartmentation such that metabolism was similar compared with plants grown at the optimum soil P level.

Long-term differences in soil P levels affected activities of ADPG pyrophosphorylase and SPS for experiment 2 (Figs. 4 and 5). Activities of both enzymes were significantly greater for the P1 treatment than for P2 and P3 during reproductive development. There was no relationship between enzyme activity (Figs. 4 and 5) and changes in starch or sucrose concentration (Figs. 2 and 3). In our earlier study with tobacco, we did not observe any relationship between ADPG pyrophosphorylase activity and starch accumulation (8). In contrast with the present study, a previous study using young soybean plants (13) deficient in P nutrition reported to increased leaf starch concentration, decreased leaf sucrose concentration, and corresponding changes in the activities of ADPG pyrophosphorylase and SPS.

The increased enzyme activity for the P1 treatment in experiment 2 may have been associated with the more favorable light environment compared with experiment 1. However, at least one other enzyme, Rubisco, was not increased by the low P treatment in experiment 2 (6). Thus, it is difficult to explain the consistently higher activities of SPS and ADPG pyrophosphorylase observed for the P1 treatment in experiment 2.

The activity of SPS (Fig. 5) declined markedly for all soil P treatments during the grain filling period, similar to leaf N, photosynthesis, Rubisco activity, and Chl (6). ADPG pyrophosphorylase activity, however, remained relatively constant over time, particularly between the second and third sampling times during which leaf senescence was occurring (Fig. 4). The maintenance of relatively constant ADPG pyrophosphorylase activity during senescence observed in this study was similar to results for aging tobacco leaves (4). These results suggest that regulation of the synthesis and/or stability of ADPG pyrophosphorylase during leaf senescence is different from other chloroplastic enzymes, particularly Calvin cycle enzymes (7, 24).

In summary, soil P treatments that had large effects on plant biomass and seed yield (5) and on the concentration of Pi in the leaves did not greatly influence leaf starch or sucrose concentrations during the grain filling period in soybean. These results were similar to a study of vegetative development in tobacco (8) and could be explained by the fact that plant growth adjusted such that leaf Pi concentration was similar for plants grown at both deficient and optimum soil P levels. Excess leaf Pi for the supraoptimum soil P treatment may have been sequestered in the vacuole such that cytosolic and chloroplastic Pi concentrations were the same as for the optimum P treatment. Our results obtained by using a soil-based culture system are contrary to other studies that indicate that P nutrition treatments markedly influence starch and sucrose accumulation and metabolism (12, 13, 18, 28, 31–33). It is possible that the method of plant culture could affect results of P nutrition studies by differentially affecting the leaf Pi concentration. Hydroponically grown plants subjected to low P levels generally experience a constantly changing environment in which solution P is depleted and added back depending upon how often the nutrient solution is
changed. Additionally, nutrient solution P would be completely in an available form for uptake, as opposed to a soil system in which P in solution remains relatively constant as determined by the properties of the soil. Therefore, under hyproionic conditions, P uptake and leaf Pi concentration may be altered by P stress treatments such that starch and sucrose metabolism are altered relative to nonstress conditions. In other cases, P nutrition treatments are instantaneously applied to plants or chloroplasts by changing the nutrient solution composition or by sequestering Pi with mannose or glycerol (2, 14, 18, 23, 28). These instantaneous treatments would be expected to alter leaf and/or organelle Pi concentration and thus influence starch and sucrose metabolism. For the soil-based culture system used in the present study, the P treatments were initiated at the time of planting with no further addition or removal of P. This culture method simulated the way that field-grown plants would experience P deficiency or supraoptimum P nutrition. Under these conditions, it can be concluded that P nutrition did not markedly influence starch and sucrose metabolism during the grain filling period in soybean.

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