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Effects of Phosphorus Deficiency on C and N Metabolism during Regrowth of Italian Ryegrass

Tae-Hwan Kim*†, Woo-Jin Jung*, Bok-Rye Lee*, Dae-Hyun Kim*, Soon-Ju Chung and Kil-Yong Kim*****

*Department of Animal Science & Institues of Agriculture Science and Technology, College of Agriculture, Chonnam National University, Kwangju 500-757, Korea
**Faculty of Applied Plant Science, College of Agriculture, Chonnam National University, Kwangju 500-757, Korea
***Department of Biological & Environmental Chemistry, College of Agriculture, Chonnam National University, Kwangju 500-757, Korea

ABSTRACT: To investigate of C and N metabolisms in response to phosphorus-deficient stress during regrowth of Italian ryegrass (*Lolium multiflorum* L.), C and N metabolites were analyzed at 0, 6, 12 and 24 days after defoliation. P-sufficient (control, +P) and P-absent (-P) nutrient solutions were applied from 7 days before defoliation, and continued for one cycle of 24 day-regrowth period. During 24 days of regrowth, dry matter of regrowing shoots and remaining tissues were not significantly different between +P and -P treatment. In remaining stubble, 70% to 91% decline of the initial level (at day 0) in all C compounds occurred during the first 6 days of regrowth. Initial amounts of nitrate and amino acids in roots were significantly higher in the +P medium. Nitrate contents in stubble in the +P medium significantly decreased for the first 12 days and then rapidly recovered, while that of the -P medium continuously decreased until day 24. Amino acids in stubble in the P medium were continuously increased during the whole regrowth period. Soluble proteins in stubble in the +P medium also largely fell down (46.0% of the initial) for only the first 6 days, however the decline in the -P medium continued until day 12. In regrowing shoots, the accumulation of C compounds was significantly higher, while that of N compounds except amino acids was largely lower in the -P medium. These results showed a stimulation of carbohydrate synthesis and a compensatory utilization of organic reserves occurred to support regrowth under P-deficient condition.

Key words **:** *Lolium multiflorum* L., Phosphorus, Regrowth, C and N metabolism

Many studies have provided evidence for the contribution of organic carbohydrate and nitrogen reserves to regrowth of new foliages in perennial forage species subjected to clipping management. Regrowth of grasses after defoliation induces the remobilization of C and N reserves. Endoamylase activity consistently increases at times of starch utilization in alfalfa taproots for wintering hardening, spring regrowth and regrowth after defoliation (Volenec *et al.*, 1991). Remobilization of nitrogen reserves actively occurred in *Lolium perenne* (Ourry *et al.*, 1988; 1989a) and in *Medicago sativa* (Kim *et al.*, 1991) during early regrowth. Translocation of organic N reserves and utilization mineral N during regrowth after shoot removal were quantified by using ^{15}N labeling (Ourry *et al.*, 1989a; Boucaud & Bigot, 1989; Kim *et al.*, 1991; Kim *et al.*, 1993).

Adequate P nutrition is important for shoot regrowth after defoliation. The influence of P nutrition on carbohydrate and nitrogen metabolism has been examined in various crop species mainly in intact plants, but the influence of P nutrition on carbohydrate and nitrogen metabolism during regrowth has not been thoroughly studied in regrowing plants. Suboptimal phosphorus supply diminishes photosynthetic CO_2-fixation rates (Brooks, 1986; Terry & Ulrich, 1973) and the expansion of photosynthetic leaf surface by decreased the hydraulic conductance of the root system (Radin & Edienbock, 1984). In soybean plants, Fredeen *et al.* (1989) showed that low-P treatment increased starch content ration in mature leaves, expanding leaves and fibrous roots; sucrose concentrations, however, were reduced by low-P in leaves and increased in roots. The increase in starch/sucrose ratio in low-P leaves was correlated primarily with changes in the total activities of enzymes of starch and sucrose metabolism (Fredeen *et al.*, 1989; Rao *et al.*, 1990).

Less is known about how P nutrition effects the C and N metabolism in remaining organs (stubble and roots). Low-P increased both starch and sucrose concentration in roots of soybean (Fredeen *et al.*, 1989). Although sucrose and hexose levels were much higher in roots of P-deficient plants, a decrease in hexose-phosphate level is often observed (Rychter

†Corresponding author: (Phone) +82-62-530-2126 (E-mail) grassl@chonnam.ac.kr
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& Randall, 1994). The effects of P nutrition on the accumulation of major C and N metabolites and use of organic reserves during regrowth period are poorly understood.

The objectives of this study were 1) to determine pool size of main C and N metabolites in remaining stubble and roots after defoliation 2) to explore the partitioning of organic reserves in response to the regrowth of shoots under P-sufficient or P-deficient nutrition.

MATERIALS AND METHODS

Plant material and growth conditions

Italian ryegrass (*Lolium multiflorum* L.) seeds were sterilized and germinated in a sand bench. When three leaf stage was developed, 5 seedlings were grown hydroponically on 3 L pot with continuous aeration. The nutrient solution was prepared as described by Kim *et al.* (1991), and renewed every 6 days. Plants were grown in growth chamber with a 18/6 h of light/dark photo period and a 25/20°C of thermoperiod. When the plants were a full-vegetative stage (about 14 weeks-old), nutrient solution was modified to give 3 mM P (P-sufficient, +P) and 0 mM P (P-deficient, -P) by substitution K_2SO_4 for KH_2PO_4. Two different P-status solutions were fed from 1 week before defoliation, anticipating the different pool sizes of organic reserves by P-nutrient treatment. Plants were then cut to a height of 6 cm above the roots base and regrowth was allowed for 24 days under +P and -P medium. Samplings were carried out on 0, 6, 12 and 24 days after defoliation with separating regrowing shoot, stubble and roots. Each tissue was frozen with liquid nitrogen and lyophilized. Tissue samples were finely ground and stored at vacuum desiccators for further analysis.

Carbohydrate analysis

Soluble sugars were extracted from about 100 mg freeze-dried samples with 92% ethanol. Tubes were shaken for 10 min at room temperature, centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was retained in 10 ml volumetric flask. The ethanol extraction was repeated more than twice, and combined supernatants. The soluble sugars contents from the supernatants were determined with Anthrone reagent (Van Handel, 1968) using glucose as a standard. The residue was dried at 80°C to remove ethanol. Deionized water was added, and heated to gelatinize the starch. The pH of the solution was adjusted to 5.1 by adding 0.2N Na-acetate buffer. Starch was digested by adding amyloglucosidase (Sigma product A3514) and α-amylase (Sigma product A0273) in the acetate buffer to each sample. Tubes were incubated at 55°C for 24 h with occasional shaking. Tubes were centrifuged as described and glucose in the supernatant was determined using glucose oxidase (Glucose Trinder, Sigma product 315-100). Starch concentrations were estimated as 0.9 × glucose concentration. Fructans present in the starch extracts was hydrolyzed with 0.1 N H_2SO_4 and fructose released quantified using resorcinol (Davis & Gander 1967). Glucose liberated from the fructan was determined as descrived, and fructan concentration was calculated as the sum of fructan glucose and fructose × 0.9.

Nitrogenous compounds analysis

About 200 mg of finely ground freeze-dried sample was extracted with 80% ethanol while heated on a hot plate for 5 min. The 80% ethanol-soluble fraction was filtered over a filter paper (Toyo-rhoshi No. 5a), centrifuged, and passed through a H^+ column (Dowex 50W× 8). The pH of one-fifth of the solution collected from the H^+ column was adjusted to pH 7.0 and this solution was concentrated to a final volume of 0.5 mL (nitrate fraction). The remaining four-fifths were passed through a Dowex 1 column (formate form). Amino acids were eluted from the Dowex 50W × 8 column with 25 ml of 0.5 N HCl and concentrated to 1.0 mL. Concentrating was done by drying each collected solution by rotary vacuum evaporation and re-dissolving the residues in distilled water to obtain the final volume of each fraction as described above. The 80% ethanol-insoluble compounds that were left on the filter paper after filtration were dried at 60°C for 24 h to obtain dry weights. One to five mg of freeze-dried powder samples were weighed (precision 10 μg) into tin capsules. The fraction of soluble protein was prepared with about 30 mg freeze-dried sample by extraction with 100 mM $NaPO_4$ buffer (pH 6.8) according to the method described by Li *et al.* (1998), and concentrated to 1.0 mL. From the liquid samples, an appropriate sample volume, usually 0.1 ml, containing more than the minimum quantities (20 μg N), was dropped into a tin capsule which had been cooled with liquid nitrogen. The tin capsules were then dried in a freeze-dryer. N determination of all fractions was performed using N single mode analysis on a ANCA-SL mass spectrometer (Europa Scientific, Crewe, UK).

RESULTS

Dry matter accumulation

Shoot regrowth of phosphorus-sufficient (+P) and phosphorus-deficient (-P) plant was not significantly different (Fig. 1A). Dry weight in regrowing shoots increased slowly for the first 6 days, thereafter increased rapidly. After 24 days of regrowth, dry weight of regrowing shoot (2.11 mg/

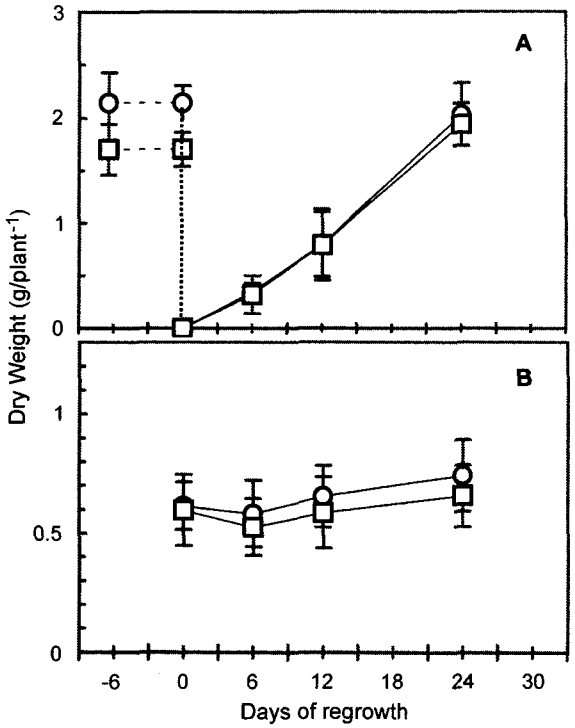

Fig. 1. Changes in dry weight in the regrowing shoots (A) and remained roots (B) during 24 days of regrowth. P-sufficient (○) and P-deficient (□) treatment were applied from 6 day before cutting, and renewed every 6 days. Dashed line indicates before defoliation. Each value is the mean ± S.E. for n=5.

plant) completely recovered the initial level (2.14 mg/plant, day 0). Dry weight of roots was less varied during entire regrowth period, and non-significant difference between +P and -P treatment was observed (Fig. 1B).

Changes in carbohydrate fractions in remaining organs

At day 0 (cutting date), when +P or -P treatment was applied previously during 1 week, total amount of carbohydrate compounds in stubble was significantly higher in the -P medium (158.3 mg/plant) than in the +P medium (139.6 mg/plant). More than 80% of total carbohydrate in two remaining tissues was stored in stubble. During the first 6 days of regrowth, a remarkable decrease (70% to 91% decline of the initial level at day 0) occurred in 3 kinds of carbohydrate fractions. When compared with the absolute amounts decreased during this regrowth period, the highest decrease appeared in soluble sugars, the second in starch, followed by fructans. It was noteworthy that most carbohydrate fractions (except starch) in roots on the -P medium decreased with a higher rate than those in the +P medium during the first 6 days.

From 6 days of regrowth, reaccumulation was so large in stubble, especially in the -P medium, that the content at day 24 recovered or exceeded the initial content (day 0) in all carbohydrate compounds in both +P and -P medium (Fig. 2A). The increasing rate of polysaccharide (starch and fructan) during this period was also higher in the -P mediumon than in the +P medium. Soluble sugars content was not significantly different between +P and -P treatment. In consequence, the contents of starch and fructan were significantly higher in the -P condition at day 24. In roots (Fig. 2B), carbohydrate contents generally followed a similar pattern with the changes in stubble (a large decrease for the first 6 days and recovery afterwards). However, a significantly higher decrease, during the first 6 days, in starch under -P condition was remarked.

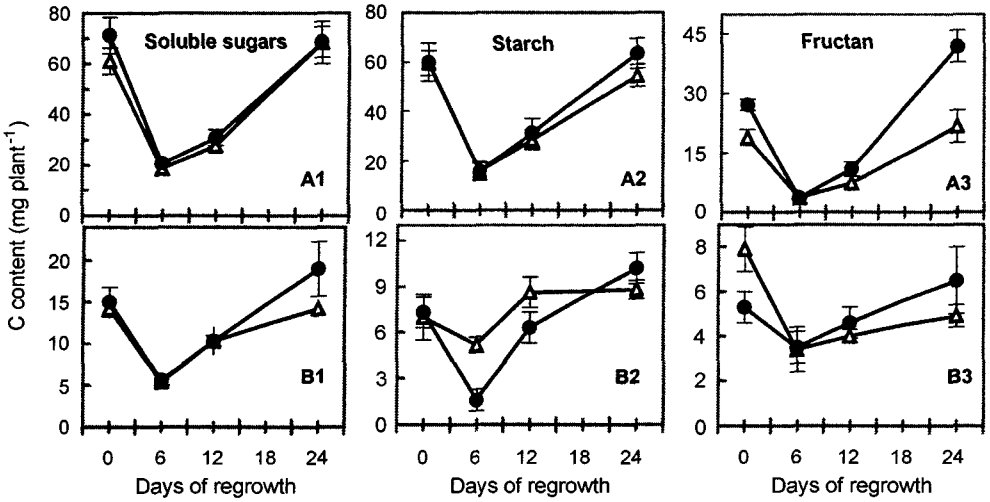

Fig. 2. Changes in soluble sugars (A1), starch (A2) and fructan (A3) contents in the remained stubble cut at 6 cm above root base and roots (B1-B3, same order) during 24 days of regrowth. P-sufficient (△) and P-deficient (●) treatment were applied from 6 day before cutting, and renewed every 6 days. Each value is the mean ± S.E. for n=5.

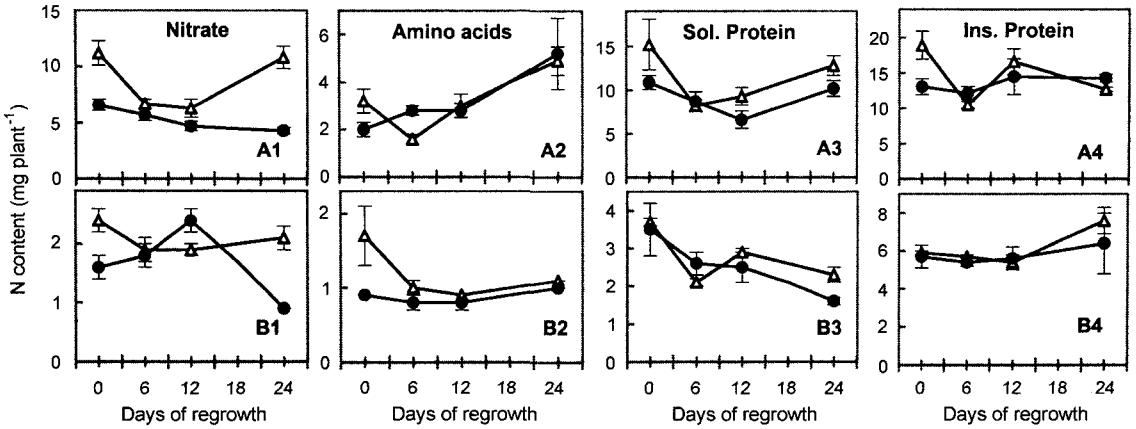

Fig. 3. Changes in nitrate (A1), amino acids (A2), soluble protein (A3) and insoluble protein (A4) contents in the remained stubble cut at 6 cm above root base and roots (B1-B4, same order) during 24 days of regrowth. P-sufficient (△) and P-deficient (●) treatment were applied from 6 day before cutting, and renewed every 6 days. Each value is the mean ± S.E. for n=5.

Changes in nitrogenous compounds in remaining organs

At day 0 (cutting day), the contents of all N compounds analyzed in stubble under +P condition were significantly higher than those of -P medium. Sum of N compounds in the +P and -P medium in this organ was 48.5 mg and 32.6 mg/plant, respectively. This indicated that N utilization and its partitioning into plant tissues, especially into reserve organs, were significantly depressed under -P condition, even though a short term (1 week) treatment was applied before defoliation. Nitrate content in stubble in the +P medium significantly decreased for the first 12 days and then highly increased, while that in the -P medium continuously decreased until day 24 without any recorvery (Fig. 3. A1). Animo acids in the +P medium greatly decreased to about half of the initial (day 0) during the first 6 days, and then rapidly recovered, whereas they continuously increased during whole regrowth period in the -P medium (Fig. 3. A2). At day 24, the content of amino acids was largely exceeded the initial level in both +P and -P medium. Soluble proteins in the +P medium also largely fell down (46.0% of the initial) for only the first 6 days, however the decline in the -P medium continued until day 12 (Fig. 3. A3). Insoluble proteins followed a general pattern in the +P medium (a great decrease for the first 6 days and a recovery thereafter), but nearly constant in the -P medium (Fig. 3. A4).

In roots, at day 0, sum of N compounds examined in both +P and -P treatment was 13.7 mg and 11.7 mg/plant, respectively. Initial reserved amounts (day 0) of nitrate and amino acids were significantly higher in the +P medium, those of soluble and insoluble protein were not significantly different between +P and -P treatment. Nitrate in this organs slightly decreased in the +P medium but significantly increased in the -P medium during the first 12 days, thereafter reversed (Fig. 3. B1). Amino acids in the +P medium significantly decreased for the first 6 days and then leveled off, while they were less varied in the -P medium (Fig. 3. B2). Soluble proteins in the +P medium also significantly decreased for the first 6 days, and then slowly recovered. However, soluble protein in the -P treatment continuously fell down from 3.5 mg at day 0 to 1.6 mg at day 24 (Fig. 3. B3). Insoluble proteins were not significantly different between +P and -P treatment for all measurements during rwgrowth period (Fig. 3. B4).

C metabolites in regrowing shoots

In regrowing shoots, the accumulation of soluble sugars was remarkably higher. From 6 days of regrowth, soluble sugars continuously increased in both +P and -P medium. After 24 days of regrowth, they were 80.9 and 94.7 mg/plant in the +P and -P medium, respectively (Fig. 4A). Starch contents were not affected by P-treatment during the first 12 days, thereafter starch accumulation was accelated in the -P condition (Fig. 4B). At day 24, starch contents in both the +P and -P medium were 13.7mg and 17.1 mg/plant, respectively. Within the first 6 days, fructan contents remained at low level and were not significantly different between +P and -P condition (Fig. 4C). A linear increase occurred in the -P medium from day 6, but a lag for the first 12 days and a sharp increase followed in the +P medium. At day 24, fructan contents in both +P and -P condition were 14.3 mg and 17.6 mg/plant, respectively.

N metabolites in regrowing shoots

Nitrate content in regrowing shoots was not significantly

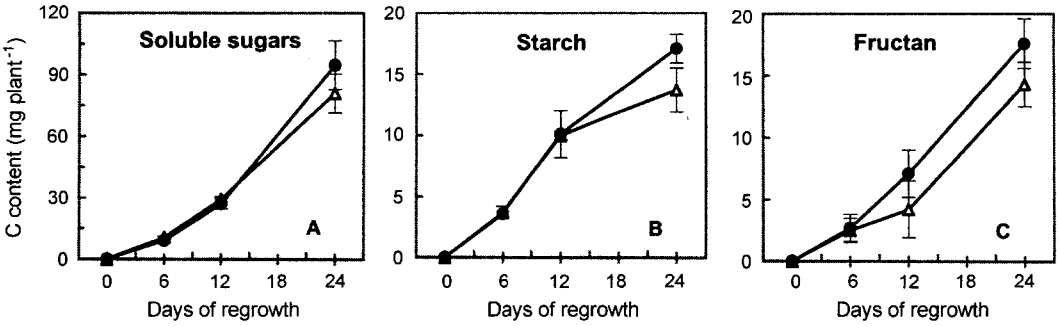

Fig. 4. Changes in soluble sugars (A), starch (B) and fructan (C) contents in the regrowing shoots cut at 6 cm above root base during 24 days of regrowth. P-sufficient (△) and P-deficient (●) treatment were applied from 6 day before cutting, and renewed every 6 days. Each value is the mean ± S.E. for n=5.

different between +P and -P treatment for the first 12 days (Fig. 4A). Thereafter, the increasing rate in the +P medium was largely higher. Amino acids were the smallest pool of N compounds examined (Fig. 4B). Amino acids content tended to be higher under -P condition, but no significant difference was shown during whole regrowth period. Soluble proteins were much sensitively responsed to -P treatment (Fig. 4C). At day 24, soluble proteins in the +P and -P medium contents were 25.6mg/plant and 17.2mg/plant, respectively. Insoluble proteins were the largest pool of N metabolites examined (Fig. 4D). There is no significant difference for the first 12 days, and then a significantly higher increase under +P medium. At day 24, the contents of insoluble proteins in the +P and -P medium were 65.7 mg and 57.5 mg/plant, respectively.

DISCUSSION

Regrowth of shoots proceeded slowly for the first 6 days after defoliation, thereafter rapidly progressed (Fig. 1A). There were little changes in dry weight of roots during regrowth period (Fig. 1B). Non-significant difference in dry weight between +P and -P treatment was observed in either regrowing shoots or roots. These results suggest that P-deficiency did not affect regrowth dynamics for one regrowth cycle of 24 days. In various intact plants, however, both leaf area and shoot dry weight were reduced by low-phosphorus nutrition, caused by a reduction in photosynthetic activity (Brooks, 1986), stomatal contactance (Brooks, 1986; Qiu & Israel, 1992) and cell expansion (Fredeen *et al.*, 1989; Rychter & Mikalsa, 1990; Li *et al.*, 1998). Non-significant difference in dry weight of regrowing shoots between +P and -P treatment (Fig. 1A) indirectly suggests that certain metabolisms might be induced to overcome P-deficient stress. In accompanied study, relatively higher uptake of anions (NO_3^-, Cl^- and SO_4^-) and cations (K^+, Ca^{+2} and Mg^{+2}) in the P medium was observed (data not shown).

More than 70% of total C and total N in two remaining tissues in both +P and -P medium were stored in stubble and the decrease of organic reserves for the early regrowth period was also higher in stubble (Fig. 2, 3), These indicate that stubble is a primary storage site of organic reserves. Ourry *et al.* (1988) observed that a large part of organic reserves accumulated in stubble of perennial ryegrass. In other forage grasses, high concentration of total nonstructural carbohydrate has been reported in stubble of timothy and switchgrass (Smith & Greenfield, 1979) and tall fescue (Volenec, 1986). All carbohydrate compounds examined in remaining tissues (stubble and roots) greatly decreased during the first 6 days of regrowth. During this period, a higher decline in soluble sugars and fructan occurred in the -P medium (Fig. 2. A1 and A3), but starch was not affected by P treatment (Fig. 2. A2). In addition, a significantly higher decrease in starch was remarked in the -P medium. These results suggested that in the P-deficient condition before defoliation induced the a higher accumulation of carbohydrate (except starch), and that degradation of reserved carbohydrates (especially in stubble) accelerated under p-deficient condition for the early regrowth period when photosynthetic activity is extremely low. Isotope studies in alfalfa demonstrated that carbohydrate reserves labeled in roots before cutting continuously decreased, whereas ^{14}C-labeled carbohydrate in the regrowing shoots increased during regrowth period (Sheard, 1973; Hodgkinson, 1969). Smith & Silva (1969) estimated that 68% of carbohydrate reserves utilized for the generation of new foliage, and a small part for the respiration. From 6 days of regrowth, all carbohydrate compounds started to recover. A higher increase and rapid reaccumulation of polysaccharide was observed in the -P medium (Fig. 2. A2-3 and B2-3).

Non-significant difference between +P and -P medium in all three C compounds in regrowing shoots for the first 12 days of regrowth (Fig. 4) indirectly indicate that the quantitative equilibrium of C metabolites, even under P-deficient

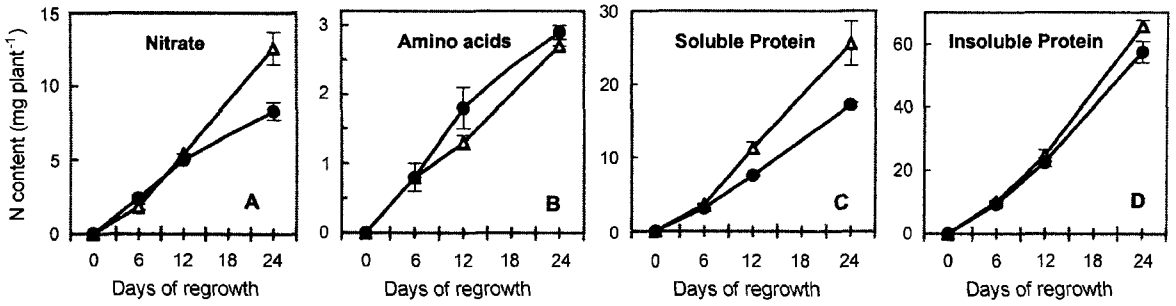

Fig. 5. Changes in nitrate (A), amino acids (B), soluble protein (C) and insoluble protein (D) contents in the regrowing shoots during 24 days of regrowth. P-sufficient (△) and P-deficient (●) treatment were applied from 6 day before cutting, and renewed every 6 days. Each value is the mean ± S.E. for n=5.

stress, possibly due to an active degradation of carbohydrate reserves during the first 6 days. This suggestion is well supported by the results of Caldwell *et al.* (1984) who gave an evidence, by ^{14}C labeling, of the translocation of carbohydrate reserves to growing shoot after the first 2 weeks of regrowth. Kim & Kim (1996) reported that the starch reserves in tap roots of alfalfa highly degraded during the first 2 weeks, with the increase of amylolytic activities.

At day 0 (cutting day), more than 70% of total N in two remaining tissues was stored in stubble. All N compounds in this organ were significantly higher in +P than -P medium (Fig. 3C). This indicated that N partitioning into plant tissues, especially into reserve organs, were significantly depressed under -P condition, even though a short term (1 week) treatment was applied before defoliation.

Under P-sufficent condition (+P), the decrease of all nitrogenous compounds in stubble remarked for the first 6 days of regrowth (Fig. 3. A1-A4), when the uptake of external N was extremely low (Ourry *et al.*, 1990; Kim *et al.*, 1991). Soil N sources (exogenous N) and organic N reserves in plant tissues (endogenous N) are both effective sources for regrowth of shoots. Kim *et al.* (1993) suggested that shoot removal stimulated N-flow from reserve organs (roots and remaining parts of shoot). Ourry *et al.* (1988) reported that the N supply for regrowth of perennial ryegrass appeared to be higher dependent on nitrogen reserves during an initial phase (0-4 days after defoliation), and then N uptake from the medium and its subsequent translocation during a second phase (4-20 days after defoliation).

Nitrate, in the -P medium, continuously decreased until day 24 without any recovery in stubble and accumulated for the first 12 days in roots, whereas in the +P medium it decreased for the first 12 days and then began to recover (Fig. 3. A1 and B1). These results suggested that under P-deficient condition nitrate assimilation in stubble much actively occurred, and that mobilization of reduced N to regrowing shoots might be accelerated to meet the demand of amino acids in regrowing shoots. The duration of soluble proteins degradation in both stubble and roots also was longer in the -P medium (Fig. 3. A3 and B3). In consequence, amino acids in regrowing shoots in the -P medium maintained at similar level with +P treatment for the first 6 days or slightly higher during later regrowth period (day 12-24). Kim *et al.* (1993) gave an evidence of active remobilization of amides and amino acids derived from N reserves via xylem for the first 10 days of regrowth of alfalfa. As shown in Fig. 4, all carbohydrate compounds in regrowing shoots in the -P medium maintained at similar level within day 12 or appeared to be significantly higher at day 24 compared to those in the +P medium. These indicate that a higher degradation of the previously accumulated C reserves before defoliation for the early regrowth period (Fig. 2. A1-A3) and a higher synthesis of starch and sucrose in expanding leaves under P-deficient stress (Rao *et al.*, 1990) are sufficient to allow the plants in the -P medium to recover completely the carbohydrate in regrowing shoots to the level of non-stressed (+P) plants.

In conclusion, a stimulation of carbohydrate synthesis and an active utilization of organic reserves under P-deficient condition successfully support for one cycle of regrowth, showing non-significant difference of dry matter production between +P and -P medium. However, this experiment does not distinguish the direct effects of P-deficiency on mobilization of organic reserves and utilization of exogenous nutrients, and does not estimate the limitation of organic reserves utilization for the subsequent defoliation-regrowth cycles.

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