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Research Article

Impacts of Phosphorus on Lignification and Carbohydrate Metabolism in Relation to Drought Stress Tolerance in Kentucky Bluegrass (*Poa pratensis* L.)

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ABSTRACT

The objective of this study was to determine effects of phosphorus on lignification and carbohydrate metabolism in Kentucky bluegrass under drought stress. Drought stress was induced by reducing of water to plants in pots. Two types of phosphorus were applied as potassium phosphate (PO_4^{3-}; P) or potassium phosphonate (PO_3^{3-}; PA) in drought-stressed plants. Drought had significant negative effects on plant growth, as revealed by reduced biomass of shoot. Drought-induced increase of lignin content was concomitant with the increase of phenylalanine ammonia-lyase (PAL). Soluble sugar content was highly increased but fructan content was largely decreased by drought stress. However, the application of phosphorus was efficient to ameliorate the adverse effects of drought. PA application improved reduced shoot growth and relative water content, and inhibited lignification synthesis with a reduction of PAL activity. P or PA application maintained soluble sugar and fructan content at similar levels to controls under drought stress. These results indicate that phosphorus application may mitigate the drought stress by inhibiting the lignification and promoting the fructan assimilation.

(**Key words**: Carbohydrates, Drought stress, Kentucky bluegrass, Lignification, Phosphorus)

Ⅰ. INTRODUCTION

Lignin is a polyphenolic polymer deposited directly in the cell wall of specialized cells and initiate contact with cellulose, which is covalently bound to other polysaccharides or proteins in the cell walls. Lignin is important for cell strength, rigidity and durability. Therefore, lignin is related to inhibition of cell expansion and plant growth. Under drought stress condition, lignin content increases in many plants such as maize (Hu et al., 2009), and white clover (Lee et al., 2007), coincident with drought-induced activation of lignifying enzymes such as peroxidases (POXs), phenylalanine ammonia-lyase (PAL), and polyphenol oxidase (PPO). POXs, which are enzymes directly involved in lignification, catalyze monolignol polymerization in cell wall. PPO catalyzes hydroxylation of monophenols and oxidation of polyphenols, and is also involved in lignification of plant cells (Rivero et al., 2001). PAL is also considered to be responsible for the phenol and lignin pathway because of catalyzing of phenylalanine to trans-cinnamate (Rivero et al., 2001; Lee et al., 2007).

Drought stress is one of limiting factors inhibiting photosynthesis due to stomatal close and decrease of CO_2 availability (Lee et al., 2008). Reduction of CO_2 fixation induced imbalance between soluble sugars and starch. In most of plants, main form of storage carbon is starch which is composed of glucose polymers. It can be rapidly mobilized to provide soluble sugars in many environmental stress conditions. Generally, drought stress leads to a depletion of starch content and the accumulation of soluble sugars in leaves (Kempa et al., 2008). Lee et al. (2008) reported that soluble sugars accumulated by water-deficit treatment are mainly due to hydrolysis of previously stored starch rather than de novo synthesis. The relationship between starch and sucrose metabolism is also regulated by inorganic phosphate (Pi) which plays a key role in translocation of triose sugars out of chloroplasts (Rychter and Rao, 2005). Pi compounds are considered to be the only form that could supply P nutrition to plants. When plants are exposed to P deficient condition, therefore, increase of starch content and decrease of sucrose content is observed in leaves of many plants including tobacco, spinach, and barley (Pieters

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et al., 2001). On the other hand, a few studies showed that P deficiency occurred simultaneous increase of starch in chloroplasts and sucrose and hexoses (fructose and glucose) in cytosol of Arabidopsis (Muller et al., 2007). Therefore, it suggested that P nutrition is closely related to carbon metabolism. Despite P is closely related to alternation of starch and sucrose metabolism in different crops, to our knowledge, its physiological significance on photosynthesis has not been fully understood on performance of Kentucky bluegrass under drought stress.

In this experiment, we hypothesized that P nutrition significantly affects the responses of lignification and carbon metabolism, so that may involve in alleviating negative impact of drought stress. To test this hypothesis, plant growth, the response of lignification-related enzymes, lignin formation, soluble sugars and starch content were examined in well-watered and drought condition with or without P supply in Kentucky bluegrass. Two types of phosphorus were applicate as potassium phosphate (P) or potassium phosphonate (PA) in drought-stressed plants.

Ⅱ. MATERIALS AND METHODS

1. Plant culture and treatments

Sods of Kentucky bluegrass (*Poa pratensis* L.) were taken from local golf (Muan CC, Chonnam, Korea) and transported to 2 L pot. These pots were cultivated in a greenhouse and watered with continuous nutrient solution regularly. After one month, 200 mL of daily irrigation per pot was supplied to well-watered treatment (control). For drought-stress treatment, 20 mL of daily irrigation containing with or without potassium phosphate (P) or potassium phosphonate (PA) was supplied. After 2 weeks, plants were harvested and separated into shoots and roots. All plant samples were stored in deep freezer (-80 °C) or freeze-dried for further analysis. Relative water content (RWC) was determined as described by Kim et al. (2004).

2. Determination of lignin content

Lignin content was determined according to the method described by Lee et al. (2007). Briefly, dried ground leaves were extracted with 95% ethanol and centrifuged at 10,000 × g for 5 min. The pellet was washed with 95% ethanol, and a mixture of ethanol and hexane (1:2, v/v). Dried pellet was mixed with acetyl bromide in acetic acid (1:3, v/v) and incubated at 70 °C for 30 min. Then, 2 M NaOH, 7.5 M NH_2OH-HCl, and acetic acid were added, and centrifuged at 10,000 × g for 5 min. The absorbance was recorded at 280 nm and calculated using lignin standard curve.

3. Lignification-related enzyme activity

Polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) activities were determined according to the method of Lee et al. (2007). Five-hundred milligrams of fresh leaves were extracted with potassium phosphate buffer (pH 7.5) containing 1mM phenylmethylsulphonyl fluoride, and centrifuged at 13,000 × g at 4 °C for 10 min. The supernatant was used for enzyme activity. PPO activity was measured by changes of catechol as substrate at 420 nm. PAL activity was determined by measuring production of cinnamic acid at 290 nm.

4. Carbohydrate analysis

Concentration of soluble sugars such as glucose, fructose, and sucrose was measured using method described previously (Kim et al., 2005). About 25 mg of finely ground sample was extracted with 1 mL of 92% (v/v) ethanol and centrifuged at 14,000 × g at 4 °C for 10 min. The glucose and sucrose concentrations were determined with anthrone reagent using glucose and sucrose as a standard, respectively. Fructose concentration was measured using fructose as a standard. For measurement of fructan concentration, the residue was incubated with starch hydrolysis enzymes at 50 °C for 24 h and then centrifuged. The supernatant was hydrolyzed with 0.1 N H_2SO_4 and neutralized with 0.1 N NaOH. Fructan concentration was calculated as the sum of fructan-glucose and fructose × 0.9.

5. Statistical analysis

A completely randomized design was utilized with three replicates for four treatments. Duncan's multiple range test was employed to compare the means of separate replicates. The significant difference was considered at $p<0.05$. All statistical

tests were performed using SAS 9.1 (SAS Institute Inc., 2002-2003).

Ⅲ. RESULTS

1. Relative water content (RWC) and biomass

Relative water content (RWC) is an important indicator of water status in plants (Lugojan and Ciulca, 2011). It was significantly decreased more than 30% due to drought stress, whereas it was maintained to control level by PA application (Fig. 1). Shoot biomass was increased by PA application under drought stress condition (Fig. 2a). However, drought stress did not affect in root biomass. It was largely increased 26% by P or PA application in drought-stressed plants (Fig. 2b).

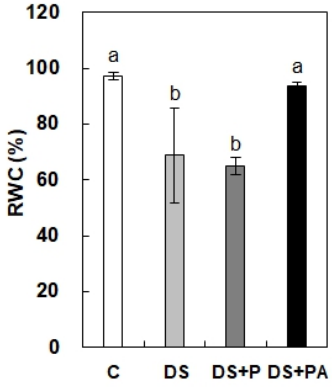

Fig. 1. Relative water content (RWC) under well-watered (control, C) and drought-stressed conditions without (DS) or with phosphate (DS+P) or phosphonate (DS+PA). Data are shown as mean ± SE for n = 3. Different letters are represented a significant difference between the means at $p<0.05$ according to Duncan's multiple range test.

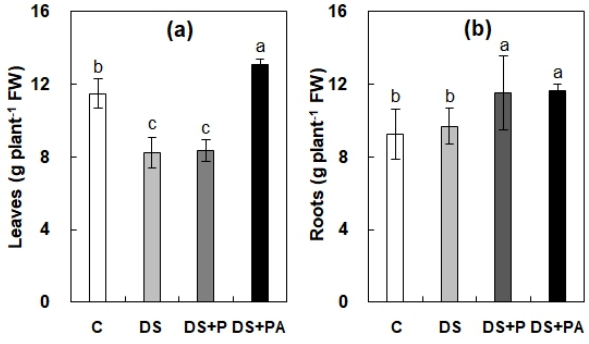

Fig. 2. Biomass in shoot (a) and root (b) under well-watered (control, C) and drought- stressed conditions without (DS) or with phosphate (DS+P) or phosphonate (DS+PA). Data are shown as mean ± SE for n = 3. Different letters are represented a significant difference between the means at $p<0.05$ according to Duncan's multiple range test.

2. Lignin concentration and activities of lignification-related enzymes

It has been well-known that drought stress-induced lignin is

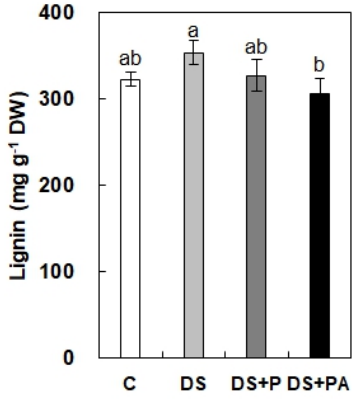

Fig. 3. Lignin content under well-watered (control, C) and drought-stressed conditions without (DS) or with phosphate (DS+P) or phosphonate (DS+PA). Data are presented as mean ± SE for n = 3. Data are shown as mean ± SE for n = 3. Different letters are represented a significant difference between the means at $p<0.05$ according to Duncan's multiple range test.

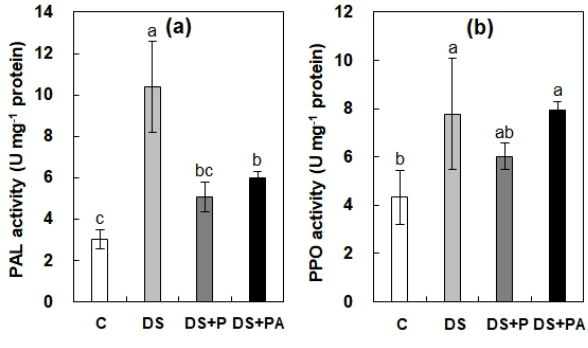

Fig. 4. Activities of PAL (a) and PPO (b) under well- watered (control, C) and drought-stressed condition without (DS) or with phosphate (DS+P) or phosphonate (DS+PA). Data are shown as mean ± SE for n = 3. Different letters are represented a significant difference between the means at $p<0.05$ according to Duncan's multiple range test.

associated with decrease of plant growth. In the present study, lignin concentration was increased +14.5% by drought stress but not significant compared to control. It was maintained or slightly decreased by P or PA application under drought stress, respectively, compared to control (Fig. 3). Phenylalanine ammonia-lyase (PAL) activity in drought-stressed plants was greatly increased by 3.1-fold compared to control, but its increase rate was much lower in P (1.5-fold higher than control) or PA (1.8-fold higher than control) applied plants (Fig. 4a). Polyphenol oxidase (PPO) activity was much higher in drought-stressed plants regardless P or PA application compared to control (Fig. 4b).

3. Soluble sugar and fructan concentration

Drought stress significantly increased concentration of soluble sugar such as glucose, and sucrose (Fig. 5a and c). Glucose concentration was 1.4-fold increased by drought stress, whereas it was maintained at control level in PA-applied plants under drought stress (Fig. 5a). Fructose and sucrose concentration were highly increased by 1.4- and 1.6-fold under drought-stressed plants. However, no significant changes in P- or PA-applied plants were observed compared to control (Fig. 5b and c). In contrast to soluble sugar concentration, fructan concentration was rapidly decreased by 21% in drought-stressed plants compared to control, while it was increased by 27% in P-applied plants under drought stress (Fig. 6).

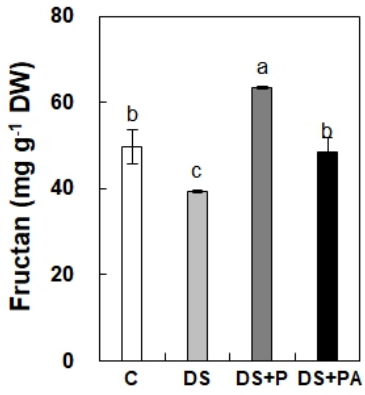

Fig. 6. Fructan concentration under well-watered (control, C) and drought-stressed conditions without (DS) or with phosphate (DS+P) or phosphonate (DS+PA). Data are shown as mean ± SE for n = 3. Different letters are represented a significant difference between the means at $p<0.05$ according to Duncan's multiple range test.

Ⅳ. DISCUSSION

Drought stress was successfully induced in Kentucky bluegrass, as the RWC in drought-stressed plants fell to a 65% of control for 14 days (Fig. 1). Low RWC would be due to low water absorption and transport ability, or high-water loss (Bittman and Simpson, 1989). In addition, a significant decrease of shoot biomass was observed in drought-stressed plants but not in roots (Fig. 2). However, phosphonate (PA)

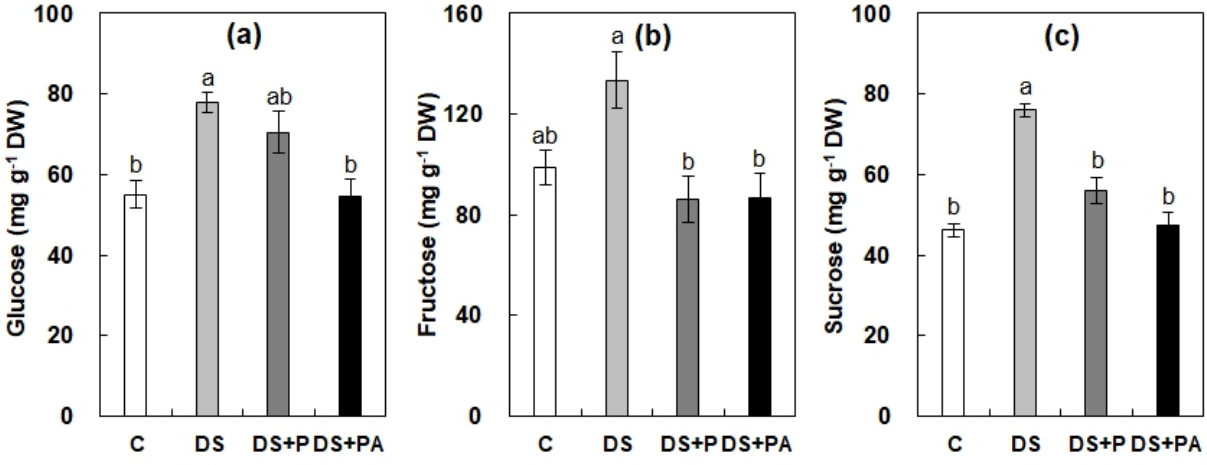

Fig. 5. Glucose (a), fructose (b) and sucrose (c) concentration under well-watered (control, C) and drought- stressed conditions without (DS) or with phosphate (DS+P) or phosphonate (DS+PA). Data are shown as mean ± SE for n = 3. Different letters are represented a significant difference between the means at $p<0.05$ according to Duncan's multiple range test.

application tended to ameliorate these negative effects. Applying P or PA applications significantly enhanced RWC, shoot and roots dry mass under drought stress (Figs. 1 and 2). Phosphorus is well known to promote root growth and stimulate tillering. Many studies reported that P supply to dry soil condition increased root hydraulic conductivity and root access to more soil water in deep soil layers, following by maintaining leaf water potential (Singh et al., 1997). In this experiment, root length was largely higher in P or PA applied plants than in non-applied control and drought-stressed plants (data not shown). These results suggest that P application may improve drought adaption by obtaining higher amount of soil water due to deep root systems.

Root growth reduction under drought stress is due to elevation of cell walls rigidity which is involved in lignification. In addition, it has been well documented that environmental stress-induced lignification occurs reduction of cell expansion, growth, nutrient content and digestibility in forage crops (Guenni et al., 2002; Lee et al., 2007). Biosynthesis of lignin is enhanced by PAL and PPO which catalyze conversion of L-phenylalanine to trans-cinnamic acid and oxidation of pholyphenols into quinnines (Boudet, 2000; Rivero et al., 2001). As expected, drought stress significantly increased activities of lignification-related enzymes such as PAL and polyphenol oxidase PPO (Fig. 4). Similar results were observed in many plants for white clover (Lee et al., 2007) and perennial ryegrass (Lee et al., 2012) under drought stress condition. In the present study, compare with drought stressed plants, PAL activity was much lower in P- or PA-applied plants under drought stress. These results indicate that phosphorus nutrition might be associated with inhibiting of lignin biosynthesis under drought stress condition. Additionally, Hernandez et al. (2007) found that P deficiency is involved in up-regulation of PAL and cinnamyl alcohol dehydrogenase genes, following by lignification of cell wall. On the other hand, Gallego-Giraldo et al. (2011) reported that transgenic alfalfa plants having low lignin levels enhanced drought tolerance. These results indicate that increase of shoot and root biomass by P or PA application is involved in decrease of cell wall lignification process due to low activities of PAL.

Drought stress-induced inhibition of shoot growth is associated with reduction in photosynthetic rate (Abid et al., 2017). In our previous work, significant decrease of photosynthesis was observed in water-deficit leaves of white clover, accompanied with reduction of CO_2 assimilation (Lee et al., 2008). Generally, drought stress-induced reduction of CO_2 fixation occurred changes of water-soluble carbohydrates (glucose, fructose, and sucrose) and storage carbohydrates (fructan and starch) concentration. Thus, drought would severely modify the integrated processes of assimilation, translocation, storage and utilization of photo-assimilated carbon. Our results showed higher concentration of soluble sugars such as glucose and sucrose in drought-stressed plants (Fig. 5a and c), as shown in tall fescue (Fu et al., 2010), and wheat (Kaur et al., 2007). Fructan concentration in response to drought stress was in direct contrast to those of soluble sugars (Fig. 6). Fructan accumulation in drought-stressed plants was significantly depressed. Similar results are reported by Spollen and Nelson (1994) that water-stressed plants resulted in a 60% reduction of fructan in tall fescue compared to control. Fructan reduction may be associated with the accumulation of sucrose, as reported by Lee et al. (2008) that the increase in sucrose accumulation is due to the degradation of starch not newly synthesized sucrose. In contrast, P or PA applied plants under drought stress increased fructan content and decreased fructose and sucrose compared to drought-stressed plants. P is known to play a central in both photosynthesis and respiration. Compounds containing P such as ATP, ADP, NAD, and NADH are key components of the Calvin cycle. Hexose produced by the Calvin cycle reacts in several steps to form an insoluble carbohydrate storage compounds called starch or fructan. In the present study, thus, P application to drought-stressed plants may promote the Calvin cycle leading to an increase of fructan. Besides, inorganic P (Pi) is involved in regulation of triose sugars transport from chloroplast which leads to an increase of carbohydrates to maintain leaf water potential under drought stress condition (Rychter and Rao, 2005). However, a significant increase of soluble sugars in P or PA applied plants under drought stress condition was not observed. Morcuende et al. (2007) reported that P limitation repressed photosynthesis which is response linked to lower demand for photosynthate resulting in higher sugar levels. These results indicate that P or PA application have a positive role in enhancing photosynthate such as fructan but not accumulate of soluble sugars, resulting in alleviation of drought stress.

In conclusion, drought stress significantly affected the RWC

and plant growth. However, P application had significant positive effects on shoot biomass, chlorophyll and RWC, resulting in inhibition of lignification accordance with decrease of PAL. In addition, fructan as a major form of carbon reserve was significantly increased by phosphorus application under drought stress with maintaining the soluble sugars. Future studies are necessary to elucidate molecular mechanisms in relation to drought stress tolerance improved by phosphorus application.

Ⅴ. ACKNOWLEGEMENT

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