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Changes in Non-Structural Carbohydrate Contents and Amylolytic Enzymes Activities during Regrowth after Cutting in *Medicago sativa* L.

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ABSTRACT : An experiment with alfalfa (*Medicago sativa* L.) plants was designed to investigate the changes in non-structural carbohydrate (NSC) contents and the activities of amylolytic enzymes during a regrowth period following defoliation. Sampling from hydroponic grown-plants were carried out at intervals during 24 days of regrowth. Shoot regrowth was very slow during the first 10 days and root growth was depressed after defoliation. Defoliation induced a great decrease in both total sugar and starch contents in taproots during the first 10~14 days. A major recovery of NSC occurred from day 15. Averaged over sampling dates, the activity of exo -amylase was about 400-fold higher than that of endo-amylase. Exo-amylase activity in defoliated plants slightly increased until day 6 (maximum level) and then decreased. Endo-amylase rapidly increased for the first 4 days after defoliation and slightly increased afterwards to a maximum on day 24. These results showed that increase in amylolytic enzyme activity in taproots coincided with the time of starch utilization during regrowth and that indicated it plays an important role in starch degradation.

Key words : Alfalfa, Regrowth, Starch, Amylolytic Activity.

Numerous studies have provided evidence for the contribution of carbohydrate and organic nitrogen to regrowth of new foliages in perennial forage species subjected to clipping management. Nelson and Smith[20], Boyce and Volenec[4], and others have shown that carbohydrate root reserves in forage species declined as new shoot growth is produced. The decline in nitrogen root reserves, however, was small as compared with the carbohydrates. Although it is generally accepted that the marked decline in root carbohydrates is positively associated with their translocation and use in the production of the new shoot

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growth, several studies with subterranean clover[17] and alfalfa[12] have suggested that the decline may be due primarily to for the use of carbohydrates in root and stubble respiration. Later authors have postulated that photosynthesis and carbohydrate reserves cannot meet the demands for regrowth immediately following defoliation, and that other factors, possibly proteins, are mobilized. The experiments using isotopic labeling have shown that constituents labeled with ^{14}C or ^{15}N prior to defoliation were redistributed to regrowing shoots of various forage species[15,21,26]. These remobilization of reserves occurred mainly for 6 days after defoliation in perennial ryegrass[22] and for 10 days in alfalfa[15].

Regrowth following defoliation in alfalfa was accompanied by a great decrease of amino acids in xylem sap[15]. Kim and Boucaud[13] also observed that the relative content of asparagin, which was the main transported form of reduced nitrogen in roots, decreased during the first 10 days of regrowth. Remobilization of protein-N coincided with the time of increase in endoprotease activity during regrowth[14]. Defoliation of alfalfa can also decreased starch concentration as much as 70% comparing with the level before defoliation[10]. Starch is the principal non-structural carbohydrate accumulated in alfalfa taproots[20]. A cyclic pattern, showing starch accumulation and degradation, occurs in taproots of periodically defoliated alfalfa plant. Although this pattern of starch metabolism has been well documented[9,11], there is limited information on the participation of enzymes involving in starch degradation during regrowth after defoliation. Amylases are of particular interest in the perennial legume alfalfa, which stores large quantities of starch in its fleshy taproot. This starch serves as a carbohydrate reserve that is used for winter survival and shoot regeneration following harvest of the shoot[5,19].

This work was initiated[1] to investigate the effect of defoliation on the contents of non-structural carbohydrate, and[2] to determine the activities of key enzymes involved in starch degradation during the regrowth period.

MATERIALS AND METHODS

1. Growth condition and experimental procedure

Alfalfa (*Medicago sativa* L. cv. Europe) seeds were sterilized and germinated in a sand bench. When primary trifoliate leaves were developed, 15 seedlings per 9 *l* culture pot were transplanted and grown hydroponically on a continuously aerated nutrient solution as previously described by Kim *et al.*[15]. Plants were grown in a growth cabinet with a 16/8 h of light/dark cycle and with a 23℃/18℃ of thermoperiod. Relative humidity maintained at 70%.

Plants were grown until the early flowering stage (about 10 weeks old), thereafter shoots were cut to a height of 6 cm above taproot level. They were then allowed to regrow and the second cutting was carried out on the 24th day of regrowth. Fresh samples were collected on 0, 1, 2, 3, 4, 6, 10, 14 and 24 days after the second cutting. Taproot tissues were lyophilized, ground to pass a 3-mm screen, and stored at −20℃ in sealed vials until analyzed. The values given in this paper, therefore, always refer to this second regrowth.

2. Carbohydrate determination

Frozen samples were extracted with boiling 80% ethanol in order to separate an ethanol-soluble fraction containing sugars (fructose,

glucose and sucrose) and the ethanol-insoluble starch. $CaCO_3$ for pH control and mannitol (mg/ml) for an internal standard were added respectively before extraction. The ethanol-soluble filtrate was evaporated under vacuum to dryness, and then dissolved to 5ml water. The ethanol-soluble sugars were separated on HPLC (Pharmacia, LKB). 20μl of alioquots were passed through on a carboohydrate analysis column (Waters Associates) with a solvent of 80% acetonitrile (flow rate, 2 ml/min.). The ethanol-extracted residue was solubilized with dimethyl sulphoxide containing 8N HCl to convert the starch to soluble form. Starch content was estimated by an enzymatic method using a combination kit of Boehringer[3].

3. Enzyme assays

Total amylase activity was determined with dinitrosalicylic acid using amylopectin[2]. Aliquots of extracts were diluted with 100 mM succinate-NaOH buffer (pH 6.0), and then incubated for 10 min at 38℃ with 1ml of 2% (w/v) amylopectin. The reaction was stopped by adding 0.5ml of dinitrosalicylic acid and boiling for 5 min. Solutions were diluted to 10ml. The *A* 540 of the solution was read using maltose as a standard. Preliminary work indicates that this assay conditions primarily measures exo-amylase (β-amylase) activity[8]. One unit of activity was defined as the release of 1μM of maltose/min.

Endo-amylase assay was carried out by a modification of procedures described by McCleary and Sheehan[18]. After an incubation of the aliquots of extracts with blocked ρ-nitrophenyl maltoheptaoside for 10min at 40℃, the reaction was stopped by adding 2ml of 20 % (w/v) trizma base. The *A* 410 of solution was measured against a deionized water blank. One unit of endo-amylase activity was expressed as liberation of 1μM of ρ-nitrophenyl/min.

RESULTS

1. Shoot and root growth

Shoots regrew slowly for 10 days after shoot removal. From 11 to 24 days after shoot removal, shoot weight increased rapidly so that total shoot weight exceeded the initial level (Fig. 1A). On the other hand, shoot weight of intact plants increased further until day 24. The intact plants were in full bloom by day 10. Roots of the shoot-removed plants grew little during the regrowth period. In intact plants dry matter of roots continuously increased and maintained much higher than those of shoot-re-

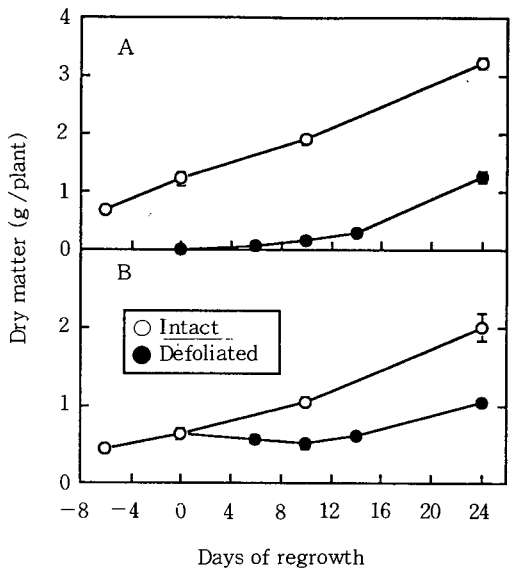

Fig. 1. Dry matter accumulation in shoot (A) and root (B) of defoliated or intact *Medicago sativa* L. Each value is the mean±SE of three replicates.

moved plants throughout the course of experiment (Fig. 1B).

2. Non-structural carbohydrate content

We have previously observed that taproots of alfalfa was an essential site of non-structural carbohydrate (NSC) storage because the contents of starch, sucrose, glucose and fructose were the highest in this tissue. Starch represents the quantitatively principal NSC in all organs considered. Taproots contained respectively 51.0% and 33.4% of total content of starch and ethanol soluble sugars[13]. From these results, special importance of this study is attached to determine the starch degradation in taproots after defoliation.

The starch content of taproots was 140.5 mg/g DM on the cutting date (day 0), and then decreased to 29.3mg/g DM during 14 days of regrowth. After the minimum level (about 20% of day 0) on the 14th day, reconstitution of starch gradually occurred. The content on the 24th day was recovered to about 52% of the initial level (d 0). In intact control plant, there was not a significant difference in taproots starch content during an experimental period (Fig. 2A). Based on the changes in starch contents, the 24 days of regrowth can be divided into starch degradation (day 0~14) and starch synthesis (day 15~24).

The total sugar (sum of ethanol-soluble mono- and disaccharides) followed a similar trend to starch, except that minimum content of sugars occurred on day 10. The reconstitution of sugars in taproots was not complete during 24 days of regrowth. On the 24th day of regrowth, the content of total sugars was 45mg/g DM (about 68% of day 0). No significant changes occurred in intact control plants (Fig. 2B).

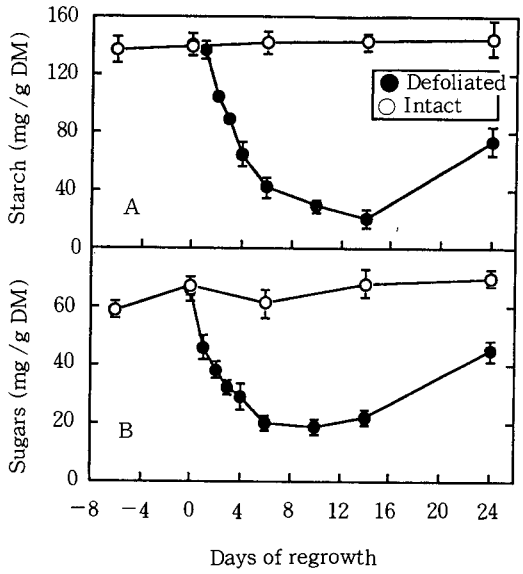

Fig. 2. Changes in starch (A) and total sugars (B) contents in taproots of defoliated or intact *Medicago sativa* L. Each value is the mean±SE of three replicates.

3. Amylolytic enzyme activities

Total amylase activity, which is due primarily to activity of exo-amylase (β-amylase)[8], increased after defoliation when expressed on a dry weight basis. The activities in taproots of defoliated plant maintained at higher levels than those of intact control plants throughout an experimental period. Exo-amylase activities rapidly increased, reaching a maximum level on day 6, and then slightly decreased, while the activities in intact plants remained little changed during an experimental period (Fig. 3A). Comparing with the starch contents, exo-amylase much early arrived to a maximum activity (day 6) than starch degradation, showing that starch was highly de-

Table 1. Changes in total starch contents and amylolytic activities in taproots of *Medicago sativa* L. during 24 days of regrowth. The values in parentheses were obtained from intact control plants. Each value is the mean±SE of three replicates

Days of Regrowth	Starch (mg/plant)		Exo-amylase (U/plant)		Endo-amylase (U/plant)	
−6		(58.8± 4.0)		(864± 37)		(1.72±0.1)
0	88.5±7.6	(88.2± 3.9)	1323± 96	(1247± 80)	2.66±0.2	(2.58±0.1)
1	84.9±6.4		1339±101		2.73±0.2	
2	62.5±5.4		1320±109		2.93±0.1	
3	52.9±3.6		1345± 84		3.48±0.2	
4	37.5±2.9		1386± 79		4.05±0.3	
6	23.3±2.1		1540±134		3.58±0.2	
10	14.6±1.6	(150.2±10.4)	1275± 77	(2195±170)	3.41±0.3	(4.37±0.3)
14	12.5±1.4		1440± 98		4.14±0.3	
24	75.5±4.9	(292.9±16.9)	2340±164	(4303±374)	8.25±0.3	(8.18±0.4)

graded until to day 14 (Fig. 2A). When temporal changes in specific activity (U/mg protein) are compared to changes in activity expressed on a tissue mass basis, similar trends are observed. Averaged over harvest dates, specific activities in defoliated and intact plants were 0.14 and 0.10 U/mg protein, respectively (data not shown).

Endo-amylase activities in taproots in defoliated plants rapidly increased for the first 4 days, and then slightly increased to a maximum at day 24 (a 190% increase over the initial level), while remaining unchanged in intact plants. Comparing to intact plants, its activity increased over twofold during 24 days of regrowth (Fig. 3B). Specific activity of endo-amylase in defoliated plants rapidly increased from 0.19 on day 0 to 0.46 U/g protein on day 10 (maximum level), and then remaining a similar level with that of day 10 (data not shown).

Changes in total starch and total amylolytic activity expressed a plant mass basis were represent in Table 1. Total starch contents greatly decreased to 85 % of the initial level during 14 days. Both of exo- and endo-amylase activities increased for the first

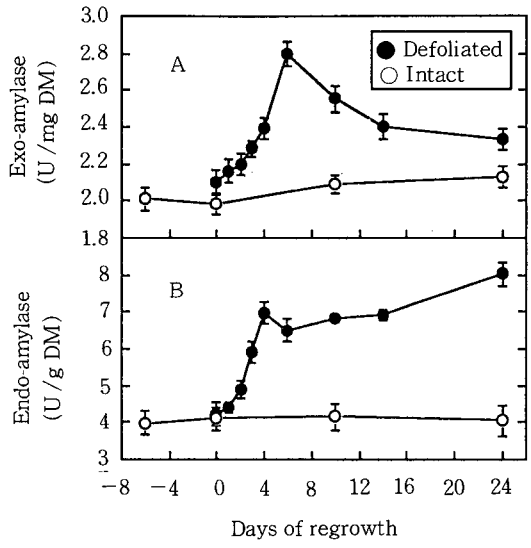

Fig. 3. Changes on exo-amylase (A) and endo-amylase (B) activities in taproots tissues of defoliated or intact *Medicago sativa* L. during 24 days of regrowth. Each value is the mean± SE of three replicates.

4~6 days. The major increase of amylolytic activity occurred from day 14 to 24, although starch degradation occurred no further. These phenomena are associated with the increase of taproots biomass during this regr-

owth period. The trends were also observed in intact control plants. Averaged over sampling dates, the activity of exo-amylase maintained at 378-fold higher than that of endo-amylase in defoliated (511-fold in intact plants).

DISCUSSION

The data obtained from this work showed that dry matter accumulation in regrowing shoot proceeded slowly for 10 days following defoliation, and then more rapidly during days 11~24. In addition, defoliation caused a depression of root growth (Fig. 1). Similar observation in alfalfa for shoot and root growth following shoot removal has been reported[6,27].

Ethanol-soluble sugars and starch contents of individual organs demonstrated that taproot was primary storage site for carbohydrate compounds and starch was the main non-structural carbohydrate (Table 1). This confirms previous observations[12,24,28]. In comparison with intact plants, it was clearly showed that defoliation induced a rapid decrease the contents of starch and sugars in taproots. The previous results of nitrogen metabolism during regrowth suggested that early regrowth was interrupted by low supply of current photosynthates observations[14,15,16]. Therefore it depends on organic reserves in source organs as C source used for the respiration and the initiation of new shoot. The starch reserves in taproots highly degraded during the first 2 weeks of regrowth, and then gradually reconstituted (Fig. 2A). From the quantitative changes in starch contents during 24 days of regrowth, a regrowth period can be divided into two phases, starch degradation (day 0~14) and starch synthesis (day 15~24). We have previously confirmed two different physiological phases in N metabolism during regrowth of alfalfa[15,16]. The first phase of regrowth was characterized by the decrease of nitrogen content in source organs and the translocation of N reserves into regrowing shoots. The second phase of regrowth was characterized by the decrease of sink strength of regrowing shoots, with increase of N uptake or N_2 fixation, with higher photosynthesis allowing reconstitution of organic reserves in source organs. Our overall results show that a common physiological pathway is involved in the reallocation of organic reserves to regrowing tissues. It should be also noted that remobization of C reserves follows a similar pattern with N remobilization during regrowth of forage species.

If internal cycling is of great importance for the economy of plant during ontogenesis process[23,25], it should be pointed out that these mechanism are basic requisites for regrowth cycle of forage species, periodically defoliated. The data obtained from this work showed that the activities of total amylase (primarily exo-amylase) in defoliated plants highly increased than those of intact plant (Fig. 3A). They also showed the kinetic differences observed between changes in starch degradation (Fig. 2A) and exo-amylase activity (Fig. 3A). It suggested that the activity of exo-amylase is not directly responsible for starch degradation. It was also observed that trends in activity of exo-amylase (β-amylase) were not consistent with its supposed role in starch degradation in taproots of overwintering alfalfa[28] and in the seeds of germinating soya bean[1]. In taproots of alfalfa, trends in total amylolytic activity

closely paralleled with concentration of soluble proteins[28]. The latter authors suggested that this enzyme might function as a storage protein, being metabolized to provide nitrogen to developing seedling. Endo-amylase activity was a minor proportion of total amylolytic activity in taproots of regrowing alfalfa. The activity of endo-amylase was 389-fold lower than that of exo-amylase when averaged over harvest dates throughout a regrowth period (Fig. 3). Doehlert and Duke[7] observed 500-fold greater specific activity of exo-amylase than endo-amylase in taproots of alfalfa cv. Sonora. It was also observed that defoliation induced an increase of endo -amylase over twofold during 24 days of regrowth. Kinetic changes in endo-amylase activity following defoliation showed a rapid increase for the first 4 days and a slight increase afterwards. (Fig. 3B). Furthermore, when expressed on a specific activity (U /mg protein), nearly 2.2-fold increase in endo-amylase activity were observed during the first 10 days following defoliation. It is possible to draw a concept that endo-amylase activity may serve an important role in initiating starch degradation. Volenec *et al.*,[28] also found that endo-amylase activity in taproots of alfalfa. consistently increased at times of starch utilization (winter hardening, spring regrowth) and concluded that it may play a major important role in starch degradation.

When expressed a plant mass basis (Table 1), the data well showed the source behavior of taproots for starch, which was characterized by a great decrease of total starch content and an increase of the activity of amylolytic enzyme during the early regrowth. We have previously discussed on the source /sink relationship for N reserves and soluble protein during regrowth following defoliation[14,15]. Although the interpretation of results comparing with other studies has been complicated by variation of genotype, tissues and the expression mode of result. we could be taken to conclude that the contribution of starch reserves to shoot regrowth is associated with the increase of amylolytic activities after defoliation.

적 요

알팔파 (*Medicago sativa* L.)의 예취후 재생기간중 저장탄수화물의 이용성을 규명하기 위해 수경재배하여 개화초기에 예취한 후 재생 24일간의 뿌리내 비구조탄수화물의 함량 및 전분 분해효소의 활력을 분석한 결과는 아래와 같다.

1. 재생초기 10일간의 잎과 줄기의 재생은 매우 느리게 진행되었으며, 예취후 뿌리의 성장이 억제되었다.
2. 예취후 초기재생 10~14일간 뿌리내 가용성 당 및 전분의 함량은 다같이 감소하였다가 이후 빠르게 회복하는 경향이었다.
3. 재생기간중 exo-amylase의 평균 활력은 endo-amylase에 비해 약 400배 이상 높았다. Exo-amylase의 활력은 재생 6일차(최고수준) 까지 증가하다가 이후 감소하였다. Endo-amylase의 활력은 재생초기 4일 동안 급격히 증가하다가 이후 재생 24일차(최고수준) 까지 서서히 증가하는 경향이었다.

이상의 결과들은 알팔파의 재생초기 동안 전분 분해효소의 활력의 증가와 아울러 뿌리내 저장탄수화물은 활발히 분해되어 새로운 조직의 재생에 이용됨을 간접적으로 제시한다.

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