Amylase Activities and Starch Contents as Affected by Source-Sink Relation during Initial Regrowth of Phasey Bean (Macroptilium lathyroides (L..) Urb.)

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Amylase Activities and Starch Contents as Affected by Source–Sink Relation during Initial Regrowth of Phasey Bean (*Macroptilium lathyroides* (L.) Urb.)

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Our objects are to determine physiological mechanisms controlling the shoot regrowth with storage starch degradation by amylases during initial regrowth of phasey bean. Plants were grown and transferred into the controlled environmental chamber set at 20 °C or 30 °C (low or high temperature pretreatment: LTP and HTP, respectively). After an 8–day adjustment period, all plants were defoliated and grown at 25 °C. Amylase activities and carbohydrate concentrations in stems and roots were compared on day 0, 1, 3, and 6 after defoliation. Starch contents at defoliation and the consumption during regrowth were higher in LTP than in HTP. As the result, new shoot production during initial 6 days was five–fold higher in LTP than in HTP, indicating the importance of the carbohydrate utilization for regrowth. Although α–amylase activity showed almost the same level between LTP and HTP, the higher starch degradation in LTP than in HTP was observed during the first 1 day. Between day 1 and 3, α–amylase activity was elevated due to a sink demand in LTP. The disappearance of the increase in α–amylase activities between day 3 and 6 may be caused by the depletion of starch but likely not by the weakening demand in sink organs.

INTRODUCTION

During regrowth of legume after defoliation, the reserved carbohydrates in residual organs were translocated into sink tissues as the substrates for respiration and biosynthetic precursor (Hodgkinson, 1969). The amount of total nonstructural carbohydrate (TNC) accumulated in the roots and crowns of alfalfa has been considered an important factor influencing regrowth after defoliation (Smith, 1962). The tetraploid population of alfalfa showed the higher accumulation of starch in taproots prior to defoliation and the more rapid shoot regrowth after defoliation relative to the diploid population (Habben and Volenec, 1991; Volenec, 1988). However, in summer, the genotype of high taproot starch concentration produced less new shoots than the low starch genotype, which may be owing to the difference in applicability to defoliation, winterhardiness or the other specific environmental stress (Boyce and Volenec, 1992; Habben and Volenec, 1990). The effect of the starch utilization on the rate of new shoot production is unclear especially in

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summer regrowth.

Starch, the main storage carbohydrate, can be hydrolyzed completely through the concerted action of α–amylase, β–amylase, debranching enzyme, and α–glucosidase. Alpha–amylase plays a major role during the degradation of native starch granules and α–glucosidase accelerates the degradation to glucose (Sun and Henson, 1990). The increase in α–amylase activity accompanied with the decrease in starch content in alfalfa taproot 4 days after defoliation suggested that α–amylase played an important role for starch hydrolysis during regrowth (Habben and Volenec, 1991). Boyce *et al.* (1992), similarly, reported the same consideration, but the negative relation between α–amylase activity and starch content occurred 10 days after defoliation. Furthermore, in taproots of sainfoin 35 days after defoliation (Kallenbach *et al.*, 1996), α–amylase activity, when assayed at 37 °C, decreased with increasing growth temperature while there was a concurrent decrease in starch content. Although the starch degradation would be necessary and important especially before the resume of photosynthesis by new leaves, the relation between starch contents and α–amylase activities was not fully investigated during initial regrowth.

The rapid changes in source–sink relations for carbohydrates occur after defoliation. Defoliation increased net photosynthesis in the remaining leaves, but CO_2 enrichment did not alter the rate of net photosynthesis in well–developed alfalfa plants (Baysdorfer and Bassham, 1985), indicating that photosynthesis following defoliation was limited by sink and so shoot regrowth may be limited by the utilization of accumulated TNC in meristems. Similarly, Suwignyo *et al.* (1995) showed that total α–amylase activities in leaves of soybean responded to modifications in the size of source and sink. It is important to consider the relation in source–sink affecting both starch contents and amylase activities.

One objective of this study is to examine the effect of accumulated carbohydrates in residual plant organs on the rate of new shoot production using the growth temperature strategy before defoliation in phasey bean, which is a tropical forage legume and often regrows in summer. Another object is to investigate the degradation of storage starch by amylases during initial regrowth.

MATERIALS AND METHODS

Plant Materials and Culture

Phasey bean (*Macroptilium lathyroides* (L.) Urb.) seeds were sown into 32–pots of 15 cm in diameter (three plants / pot) filled with an 1:1 (v/v) mixed soil of vermiculite and compost containing 200 mg/L of N, 700 mg/L of P and 100 mg/L of K on June 17, 1999. Plants were grown for 34 days in the experimental field of Kyushu University, randomly divided into two groups and then transferred into the controlled environmental chamber (Biotron institute, Kyushu University) set at 20 °C (low temperature pretreatment: LTP) or 30 °C (high temperature pretreatment: HTP) with 70% relative humidity. After an 8–day adjustment period, all plants were cut at 10–cm height above the soil surface, and followed by removing the leaves and the petioles from the remaining plants being 10–cm in height. Both the groups were immediately transferred into the controlled chambers of 25 °C.

Organ Sampling

Four pots *per* temperature treatment were sampled immediately following defoliation and 1, 3 and 6 days later at 6 p.m., respectively. The stems (above the soil surface) were separated from the new shoots (the lateral buds, petioles, and leaves), and the roots except the fine roots (< 1 mm diameter) were obtained to the depth of 10 cm. The samples were immediately packed in dry ice and stored at -20 °C. All samples were lyophilized, removed from soil and nodules, and then ground to pass a 1-mm aperture screen.

Carbohydrate Determinations

The protocol of carbohydrate determinations was based on the previous report (Asano *et al.*, 2000). Carbohydrates were extracted with 80% (v/v) ethanol. Mono- and disaccharide solutions prepared from supernatant of the extraction were analyzed by HPLC system, and starch from the residual pellet was hydrolyzed to glucose and assayed using a coupled glucose oxidase-peroxidase system.

Enzyme assays

Crude extracts were prepared as previously described (Asano *et al.*, 2000). Total amylase activities were determined by measuring reducing power released following hydrolysis of soluble starch (Gallagher *et al.*, 1997) with DNS color reaction. Alpha-amylase and α-glucosidase activities were assayed using the procedure described by McCleary and Sheehan (1987) and Gallagher *et al.* (1997), respectively, and incubation temperature was the same as that of growth (20, 25 or 30 °C).

RESULTS AND DISCUSSION

Effects of pretreatment

Temperature adjustments of 20 °C or 30 °C before defoliation significantly modified the dry weights and glucose and starch contents in both organs, which were elevated by low temperature (Table 1). Consequently, TNC contents (estimated from the sum of fructose, glucose, sucrose and starch contents) in LTP were approximately 12% in both

Table 1. Effects of the temperature pretreatment of 20 °C or 30 °C on dry weight, fructose, glucose, sucrose, and starch contents, and total amylase, alpha-amylase and alpha-glucosidase activities in stem and root organs at defoliation.

	Stem			Root		
Measurement	20 °C	30 °C	P	20 °C	30 °C	P
Dry weight (mg/plant)	529	449	0.008	430	306	0.034
Fructose (mg/g DW)	9.6	7.8	0.230	6.5	7.1	0.529
Glucose (mg/g DW)	21.9	11.5	0.025	15.9	7.7	0.026
Sucrose (mg/g DW)	12.4	11.6	0.663	34.3	24.6	0.101
Starch (mg/g DW)	77.2	9.2	<0.001	62.6	11.3	<0.001
Total-amylase (nmol/min/g DW)	250	260	0.878	287	421	0.358
Alpha-amylase (nmol/min/g DW)	5.9	21.7	0.003	7.7	12.7	0.104
Alpha-glucsidase (nmol/min/g DW)	1.6	4.4	0.005	3.0	6.1	<0.001

organs, which was two- to three-fold higher than in HTP. Lower temperature increased the dry weight of white clover stolon with a concurrent accumulation of TNC to adapt to winter survival (Boller and Nösberger, 1983), in relation to starch deposition by the function of soluble starch synthase (Denyer *et al.*, 1994). In addition, higher temperature increased the shoot and root respiration in alfalfa with the result of the decrease in net accumulation of stored carbohydrates regardless of the increase in net photosynthesis (Al-Hamdani and Todd, 1990). Therefore, low growth temperature induced the accumulation of storage carbohydrates and other nutrients followed by the increase in dry weights of phasey bean organs.

Temperature adjustment also influenced some amylase activities. The activities of α-amylase in stems and α-glucosidase in both organs were significantly ($P<0.01$) higher at 30 °C than at 20 °C when assayed at each growth temperature (Table 1). These differences may be caused by a response to the stronger demand of sink tissues due to the acceleration of respiration with increasing temperature (Al-Hamdani and Todd, 1990). The continuous higher α-amylase activities for 8 days could support the result of low starch contents less than 2% in HTP, whereas the lower α-amylase activities in LTP resulted in starch accumulation more than 5% where the negative correlation ($r=-0.751$, $n=8$) between starch content and α-amylase activity was obtained (data were not shown). Alpha-glucosidase accelerates the degradation of starch granules (Sun and Henson, 1990), and the higher α-glucosidase activities in the present experiment supported the low starch accumulation in HTP. However, the negative correlation ($r=-0.63$, $n=16$) between the α-glucosidase activity supplying glucoses and glucose content at the end of the temperature pretreatment suggested that the enzymatic supply by α-glucosidase catalysis did not control glucose contents.

Regrowth after defoliation

New shoot production clearly began between day 1 and 3, and then rapidly increased after 3 days (Table 2). The utilization of TNC accumulated in the roots of alfalfa (Hodgkinson, 1969; Smith, 1962) and in the stubble of perennial ryegrass (Donaghy and Fulkerson, 1997) has been considered an important factor influencing the shoot regrowth after defoliation. Similarly, the regrowth of phasey bean developed remarkably faster in the plants in LTP, hence the results supported the consideration that the rate of new shoot production depended in part on the storing TNC contents of remaining shoots and roots in the early stage of regrowth.

Table 2. Dry matter of new shoots in the temperature pretreatment of 20 or 30 °C following defoliation (n=5).

	New shoots production (mg/plant)			
Pretreatment	Day 3	s.e.	Day 6	s.e.
20 °C	48	± 2.9	134	± 3.1
30 °C	11	± 2.0	25	± 1.9

Storage carbohydrates utilization during the first 1 day after defoliation

The significant changes in total amylase and α-amylase activities were not detected between day 0 and 1, but the α-amylase activities in LTP tended to rise due to the increase in growth temperature from 20 to 25 °C (Table 3). The increase in growth temperature also promoted the α-glucosidase activities in stem and root ($P<0.01$ and <0.05, respectively) in LTP (Table 3). Alpha-amylase gene expression in suspension-cultured cells was induced by the deprivation of carbohydrate nutrient with a lag period of 2–4 h (Yu *et al.*, 1991). The deprivation of carbohydrates in LTP did not occur

Table 3. Fluctuation of amylase activities in total amylase, alpha-amylase, and alpha-glucosidase activities at high (30 °C: HTP) or low (20 °C: LTP) temperature pretreatment in stem and root organs.

		Total amylase		Alpha-amylase		Alpha-glucosidase	
Organs	Day	LTP	HTP	LTP	HTP	LTP	HTP
				nmol/min/g DW			
Stem	0	250	260	5.9	21.7	1.58	4.45
	1	216	307	16.5	20.8	4.64	4.04
	3	456	245	47.1	23.6	5.11	2.86
	6	382	150	41.8	23.5	2.99	2.50
	PLSD†	138	NS‡	20.4	NS	2.02	NS
Root	0	287	421	7.7	12.7	2.99	6.14
	1	326	182	11.1	10.1	5.23	5.56
	3	589	254	40.6	16.2	5.43	5.32
	6	141	152	18.9	22.5	3.87	3.91
	PLSD	428	NS	28.1	NS	1.83	1.23

† Fisher's protected least significant difference ($P<0.05$). ‡ not significant.

Table 4. Fluctuation of carbohydrate contents in fructose, glucose, sucrose, and starch at high (30 °C: HTP) or low (20 °C: LTP) temperature pretreatment in stem and root organs.

		Fructose		Glucose		Sucrose		Starch	
Organs	Day	LTP	HTP	LTP	HTP	LTP	HTP	LTP	HTP
					mg/g DW				
Stem	0	9.6	7.8	21.9	11.5	12.4	11.6	77.2	9.2
	1	7.2	6.1	13.3	3.0	14.9	6.1	66.2	7.5
	3	11.3	5.6	12.2	4.4	8.8	5.3	40.6	9.1
	6	10.4	6.6	4.6	2.4	3.8	3.8	7.7	4.9
	PLSD†	NS‡	NS	4.4	6.6	2.2	3.0	27.1	NS
Roots	0	6.5	7.1	15.9	7.7	34.3	24.6	62.6	11.3
	1	6.1	4.2	7.3	4.7	21.4	8.6	33.0	7.4
	3	7.2	5.5	8.3	2.9	8.7	4.1	9.3	4.0
	6	4.9	5.4	2.1	0.8	5.0	3.5	3.6	4.1
	PLSD	1.5	NS	5.0	NS	12.2	7.3	16.5	NS

† Fisher's protected least significant difference ($P<0.05$). ‡ not significant.

in the remaining organs of phasey bean. The α-amylase activities in HTP, where sugars were almost depleted, may show no significant changes due to the decrease in the growth temperature from 30 to 25 °C. The results by Gallagher *et al.* (1997) showed no changes in α-amylase activity during 1 day after defoliation in young stolon of white clover while storage carbohydrates decreased. Therefore, the net activities of starch degradation enzyme should not generally change during 1 day after defoliation. Alpha-amylase is also an important enzyme controlling starch hydrolysis in source organ, supported by many reports (Boyce *et al.*, 1992; Gallagher *et al.*, 1997; Habben and Volenec, 1991; Volenec *et al.*, 1991). However, the α-amylase and α-glucosidase activities showed almost the same level between LTP and HTP, respectively, and the 35 (mg/gDW) higher starch degradation in LTP than in HTP was observed (Tables 3 and 4). In addition, the starch contents in LTP were still largely higher than those in HTP, though starch content only in the root in LTP decreased significantly ($P<0.01$) (Table 4). Therefore, the results of variation in amylase activities and starch contents in this experiment indicated that the control of starch degradation rate during the first 1 day was affected by other factors such as substrate (starch) contents rather than amylase activities.

Sucrose contents in HTP significantly ($P<0.01$) decreased in both organs, but in LTP, increased significantly ($P<0.05$) in the stem and decreased in the root. Glucose contents decreased in all compartments and the decreases were significant in both organs in LTP and in the stem in HTP ($P<0.01$ and $P<0.05$, respectively) (Table 4). Similarly, considerable disappearances of sugars were observed in stolons or roots of white clover (Gordon *et al.*, 1986; Baur-Höch *et al.*, 1990) during 1 or 2 days of regrowth. Sugars were also intermediates via starch degradation, and the contents will be controlled by the interaction between the supply from starch degradation and the demands for respiratory energy and biosynthetic precursor. Therefore, the consumption of sugar for sink tissues was over the supply of sugar from starch degradation and sugar contents decreased, which was an inevitable result.

It was likely to supply higher sugar from starch degradation and to consume more carbohydrates in LTP compared with HTP.

Storage carbohydrates utilization between day 1 and 3 after defoliation

The axillary buds began to differentiate and grew between day 1 and 3 after defoliation (Table 2) and some of them in LTP then expanded their leaves by the sampling at day 3. The TNC contents continued to decrease in LTP and showed nonsignificant changes in HTP. In detail, sucrose contents in LTP significantly ($P<0.05$) decreased in both organs and starch contents in LTP decreased in stems ($P=0.06$) and roots ($P<0.01$) (Table 4). The supply of more carbohydrate to the growing axillary buds in LTP than in HTP through the 3 days might have occurred and induced four-fold new shoot production in LTP as much as that in HTP. Baur-Höch *et al.* (1990) showed the same results, where the decrease in the storage carbohydrates contents was proportional to their initial contents in white clover stolons and there was the concomitant production of leaf dry matter after defoliation. The expanding new leaf showed the recover of carbon fixation to a certain degree but the demand for carbohydrates in LTP would still exist because the decrease in TNC continued. The decreased starch content and the depleted sucrose suggested the relative shortage of supplying carbohydrates during the period of day 1 to 3

compared with the first 1 day. The size of source and sink affected total α–amylase activities in leaves of soybean (Suwignyo *et al.*, 1995). Yu *et al.* (1991) showed that the sucrose deprivation induced the expression of α–amylase genes in suspension–cultured cells of rice. It is supposed that as the result of a compensation for such decreasing supply, total amylase and α–amylase activities in each stem were elevated significantly ($P<0.01$) in LTP (Table 3). The above–mentioned consideration agrees that α–amylase plays an important role in the hydrolysis of starch during regrowth (Boyce *et al.*, 1992; Habben and Volenec, 1991; Volenec *et al.*, 1991).

Storage carbohydrates utilization between day 3 and 6 after defoliation

Only in LTP, the significant decreases were shown in glucose ($P<0.01$), sucrose ($P<0.001$) and starch ($P<0.05$) contents in stems, and fructose ($P<0.01$) and glucose ($P<0.05$) in roots (Table 4). Consequently, TNC contents showed larger decrease in LTP than in HTP where there was a slight decrease, and the new shoot production in LTP was above five times as high as that in HTP (Table 2). The regrowing organs including new shoots should still play as a stronger sink in LTP than in HTP while the photosynthate was already supplied to some extent. Furthermore, the starch contents had already depleted by the sampling day of 6 and there was not significant ($P<0.05$) increase in α–amylase activities (Table 3). Gallagher *et al.* (1997) showed almost the same result as that in young and old stolon tissues of white clover 8 days after defoliation. The depletion of starch, but likely not the weakening demand in sink organs, may inhibit the increase in α–amylase activities. The α–glucosidase activities decreased in the root in HTP and in the stem in LTP significantly ($P<0.05$) (Table 3), which might be regulated by the depletion of starch or sugars earlier than that in the case of α–amylase and the mechanism remains to be clarified.

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