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Carbon and nitrogen reserves of leafy spurge (*Euphorbia esula*) roots as related to overwintering strategy[1]

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Abstract:

Leafy spurge (*Euphorbia esula* L.), a serious perennial weed of temperature range and pasture lands, has continued to colonize despite various control strategies. The persistence of this species can be attributed in part to the presence of an extensive root system containing abundant organic reserves. These components, established towards the end of the growing season, are remobilized to support early spring growth. Carbohydrates comprise the bulk of reserve material with late fall increments in free sugars being associated with reductions in starch content. Nitrogenous components undergo significant seasonal fluxes, with free amino acids and soluble proteins reaching maxima during late fall. Asparagine, glutamic acid, serine, ornithine, proline, arginine and aspartic acid all contribute significantly to the storage of nitrogen. Changes in nitrate content are associated with the overwintering process. These observations are indicative of the role that nitrogen plays in the overwintering strategy and regenerative capacity of leafy spurge roots.

Keywords:

Amino acids, carbohydrates, *Euphorbia esula*, nitrate, protein, roots, storage.

Abbreviations:

DDW, double-distilled water; GABA, γ-aminobutyric acid; HPLC, high performance liquid chromatography; TNC, total non-structural carbohydrate.

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Introduction

Leafy spurge, a Eurasian introduction, first appeared in North America during the early 19th century (Evans and Torell 1986, Lym *et al*. 1986). Since then, infestations have been identified in 6 Canadian provinces and 26 American states, the most serious in midwest regions (Nissen and Foley 1987). The persistence and spread of this perennial have been attributed to several factors. Plants can tolerate a wide variety of habitats and environmental conditions and readily establish dense stands (Morrow 1979, Steenhagen and Zimdahl 1979). Attempts to eradicate established populations of leafy spurge have met with limited success due to the existence of a deep and extensive root system (Best *et al*. 1980, Lym and Messersmith 1987). Such efforts have been additionally hampered by its high regenerative capacity. Regrowth has been recorded for relatively small root fragments buried at considerable soil depths (Coupland and Alex 1955). Production of new shoots occurs from crown buds formed during fall at the soil surface, as well as from numerous adventitious buds located near the surface along much of the horizontal and vertical components of the root system (Best *et al*. 1980, Nissen and Foley 1987). This species also is effectively disseminated through the production of seed from mid-summer to late fall (Best *et al*. 1980). Leafy spurge exhibits allelopathic tendencies (Steenhagen and Zimdahl 1979) and possesses latex-containing substances which are toxic to cattle, horses and sheep (Lym *et al*. 1986, Lym and Kirby 1987).

Some biochemical characterization of leafy spurge has been conducted, although emphasis has been placed primarily on carbohydrates (LeTourneau 1956, Bybee 1979, Lym and Messersmith 1987). There is little information available regarding metabolism in roots as it pertains to storage and vigour. Carbohydrates and/or lipids, and nitrogenous compounds are the major reserves in perennials. In many roots, carbohydrates predominate quantitatively as storage reserves and form a primary source of reserve energy (Heilmeier *et al* 1986).

Nitrogen may be as significant qualitatively with regards to overall storage strategy (Suzuki and Kohno 1987). Seeds sequester storage nitrogen predominantly as proteinaceous deposits. However, reserve nitrogen has been observed to be partioned into nitrate amino acids and/or proteins in roots of perennials (D. R. Cyr and J. D. Bewley, unpublished data). A distinction also can be made between storage and accumulation of nitrogen (Millard 1988).

The seasonal flux of nitrogenous components in the roots of leafy spurge and their contributions to regenerative capacity have not been adequately addressed. Potentially, any factor which interferes with deposition of these reserve components could severely affect the ability of the weed to compete in the succeeding growth season. This raises the potential of improved application of biological control, herbicide and defoliation techniques.

Materials and methods

Plant material

Samples were collected randomly from a weed garden at the Univ. of Guelph on a monthly basis at approximately midday. A minimum of 5 plants was sampled, each representing a single replicate. Following collection, roots were rinsed in deionized water and surface-dried. Samples for fresh weight and dry weight determinations were weighed immediately prior to drying to a constant weight at 65-70°C. For biochemical analyses, samples were quickly immersed in liquid nitrogen, lyophilized for 48 hours, ground to 40-mesh and stored at -20°C.

Total soluble carbohydrates

Free sugars were extracted with 80% ethanol for 2 hours at 95-100°C prior to being assayed colourimetrically using the phenol-sulphuric acid method of Liu *et al*. (1973).

HPLC analysis of soluble carbohydrates

A 10-ml sample of the ethanol extract containing 1.5-15 mg of free sugars and 1 m*M* mannitol as the internal standard was sequentially passed through Waters C_{18}, and Alumina A SEP-PAKs (Waters, Milford, MA, USA) to remove lipid and protein, and salts and acids, respectively. After discarding the first 2.5 ml, a volume of eluant (7.5 ml) was collected and dried in a Savant SpeedVac (Savant, Farmingdale, NY, USA), resuspended in 1.5 ml double-distilled water (DDW) and passed through a 0.5 mm Millipore FH Filter (Nihon Millipore, Kogyo K.K., Yawa, Japan) prior to injection on an HPLC. A 20-µl sample containing 15-150 µg of sugars was injected into a Perkin-Elmer Series 2 Liquid Chromatograph equipped with a 300 × 6.5 mm (i.d.) Waters Sugar-Pak 1 column protected with an Ion-Guard Cartridge (Interaction Chemicals, Mountain View, CA, USA). Components were eluted isocratically at 0.3 ml min^{-1} at 90°C using degassed 50 mg l^{-1} Ca-propionate as the mobile phase (filtered through a 0.2 µm Millipore membrane filter), and analyzed using a Gilson Model 131 Refractive Index Detector and a Perkin-Elmer Sigma 10B Chromatography Station. All reagents were obtained from Sigma (St. Louis, MO, USA).

Starch

Starch was extracted in 52% (w/v) $HClO_4$ for 30 minutes as described by Hassid and Neufeld (1964) and quantified as the starch-iodine complex (Siminovitch *et al*. 1953).

Total free amino acids

Free amino acids were estimated with ninhydrin according to Moore and Stein (1948) following extraction in 10% (v/v) ethanol for 1 hour.

GLC analysis of free amino acids

Individual amino acids were analyzed by gas-liquid chromatography, as their *t*-butyldimethylsilyl derivatives, utilizing procedures and equipment described by Goh *et al*. (1987).

Soluble protein

Soluble proteins were extracted using procedures modified from Rutherford and Deacon (1972) and quantified by the Bradford (1976) method using bovine serum albumin as a standard. Samples were extracted at high speed on a vortex for 1 minute at 4°C in 0.1 *M* sodium phosphate, pH 6.8, containing 10 m*M* cysteine and 1 g (g dry weight)$^{-1}$ of insoluble polyvinylpolypyrrolidone. Protease activity was inhibited by addition of 10 m*M* phenylmethylsulfonyl fluoride and 10 m*M* leupeptin immediately prior to extraction.

Nitrate + nitrite

Nitrate + nitrite were extracted in DDW for 15 minutes and quantified on a Technicon AutoAnalyser II (Technicon, Tarrytown, NY, USA) using the diazotization protocol (Kamphake *et al*. 1967).

Results and discussion

In temperate species, the importance of storage reserves of overwintering tissues in relation to the capacity for spring regrowth has been recognized for a long time. Nonetheless, investigations of reserve metabolism in perennials have been relatively narrow in scope. In biennial and perennial plants, carbohydrates constitute the major volume of reserve material (Lym and Messersmith 1987). Excess photosynthetically fixed carbon in temperate species is stored as non-structural polysaccharides in perennating organs during fall, hydrolyzed to monosaccharides and disaccharides during winter dormancy, and then redistributed in the spring to be utilized as respiratory substrates for growth prior to the acquisition of net photosynthetic activity.

The dry matter ratio of leafy spurge roots increased from spring minima to peak levels by early fall, a pattern strongly correlated with shifts in carbohydrates (Fig. 1). Thus, elevated levels of starch coincided with peak dry weights and peak spring accumulation of soluble carbohydrate coincided with maximal water contents. As observed in other perennials (Lym and Messersmith 1987), starch accumulation was most pronounced subsequent to flowering and seed set. Increments in soluble carbohydrates during late fall and early winter were concurrent with the decline in starch.

Sucrose is the primary component of the soluble pool, both qualitatively (Lym and Messersmith 1987) and quantitatively (Fig. 1). Lym and Messersmith (1987) also detected trace amounts of maltose and fructose. In contrast, we observed small winter increments in glucose, and fructose levels remained at or near detection limits throughout the year. The latter distribution of sugars is in close agreement with the analyses of leaves

and stems by LeTourneau (1956). It is likely that sucrose serves as a highly mobile substrate for supporting rapid growth during early spring, although it has also been suggested that it is important in the development of winter hardiness of leafy spurge roots as well as elongation and winter survival of root buds (Lym and Messersmith 1987). Sakai and Yoshida (1968) have recognized cryoprotective roles for sugars and other small colligative biomolecules in plant tissues.

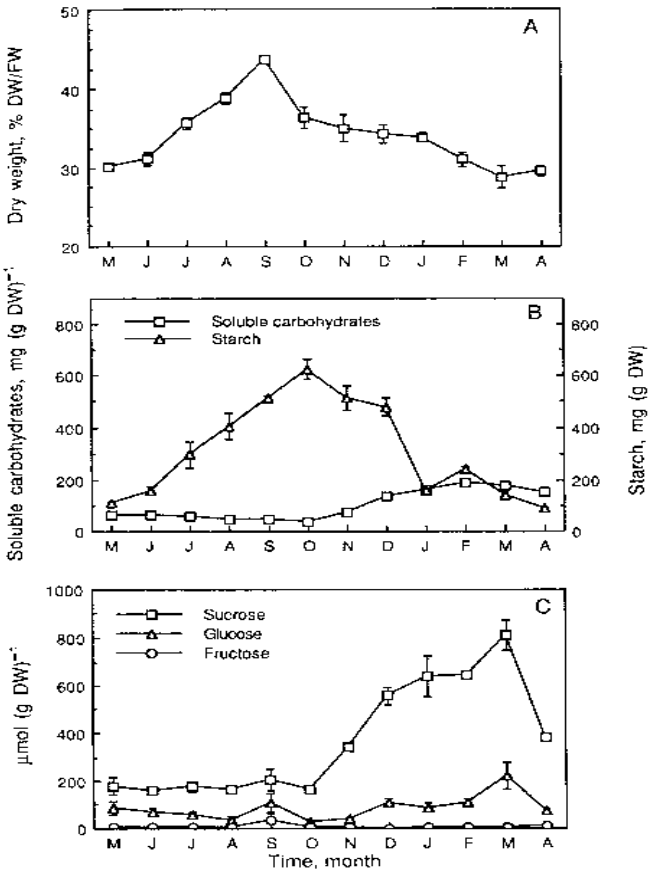

Fig. 1. Monthly variation of (A) % dry weights of leafy spurge roots. Values represent means ± SE of 5 replicates. (B) Total soluble carbohydrate and starch content of leafy spurge roots. Values represent means ± SE of 5 replicates. (C) Sucrose, glucose and fructose content of leafy spurge roots. Values represent means ± SE of 3 replicates.

Quantitatively, the decline in starch was greater than the increase in total free sugars in spring (Fig. 1). Maintenance respiration during overwintering probably accounts for a significant proportion of the carbon lost from the storage carbohydrate pool. Heilmeier *et al*. (1986) have observed that the rootstock of *Arctium tomentosum* experiences at 25% loss of carbon during this time. Redistribution of carbon could also have occurred through the provision of carbon skeletons, reducing equivalents and ATP through respiratory pathways for production of nitrate + nitrite, amino acids and proteins (Oaks 1986;

Fig. 2). Trends of carbohydrates and nitrogenous components remained similar when expressed on a fresh weight basis (data not shown).

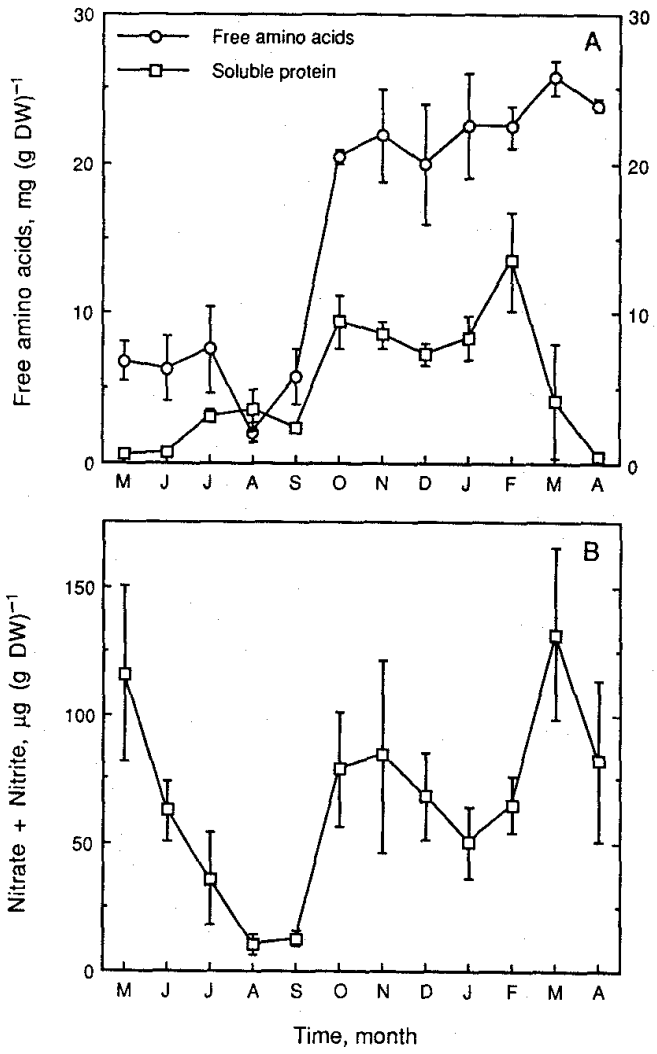

Fig. 2. Monthly variation of (A) Total free amino acid and soluble protein content of leafy spurge roots. Values represent means ± SE of 5 replicates. (B) NO_3^- + NO_2^- levels in leafy spurge roots. Values represent means ± SE of 5 replicates.

Recent studies have led to the proposal that nitrogen is the critical currency of storage metabolism; nitrogen is conserved during overwintering and is cycled extensively during the growing season (Agren and Ingestad 1987). In addition, Heilmeier *et al.* (1986) have found that the proportion of stored nitrogen remobilized for early spring growth was considerably higher than that of carbon. Thus, carbohydrate reserves appear to accumulate in considerable excess. Weed control programs, however, have traditionally assumed that regrowth capacity is primarily dependent on the status of carbohydrate reserves; therefore

nitrogenous reserves have largely been ignored. Because of the abundance of storage carbohydrate, control measures targeted at this facet of metabolism often have become cost and time ineffective.

One or more of nitrate, amino acids and proteins can function in nitrogen storage (Millard 1988). In leafy spurge roots, all three components underwent extensive changes prior to the onset of winter (Fig. 2). Even at maximum, amino acids and proteins were present in much lower amounts than carbohydrates, although the rapid increases in total amino acids were recorded earlier than those of soluble carbohydrates. Amounts subsequently remained high throughout the winter. These seasonal fluctuations in accumulation of amino acids and soluble protein (Fig. 2) are strikingly similar to those observed in two herbaceous perennials from the Compositae, chicory and dandelion (D. R. Cyr and J. D. Bewley, unpublished data).

Fall increments occurred for most of the individual free amino acids (Fig. 3). Much of the increase in the pool was accounted for by those amino acids most commonly associated with nitrogen storage, viz. aspartic acid, asparagine, glutamic acid, proline and arginine. With the exception of glutamine, these results closely resemble patterns observed in chicory and dandelion roots (D. R. Cyr and J. D. Bewley, unpublished data). Asparagine, glutamic acid and serine exhibited the largest changes, with increases of up to 10-fold over summer minima. Somewhat smaller increases were noted for alanine, cysteine, isoleucine and lysine. Maximum contents of the other protein amino acids did not exceed 3 μmol (g dry weight)$^{-1}$ and only minor fluctuations in their contents were observed (data not presented). Citrulline, γ-aminobutyric acid (GABA) and hydroxyproline were not detected in the soluble pool; however, ornithine, a non-protein amino acid, displayed a marked increase during the winter months similar in magnitude to that of asparagine. Although there are no reports of the presence of ornithine, GABA has been detected in leafy spurge foliage (LeTourneau 1956).

Arginine is often a dominant storage component of the free amino acid pool, although winter composition of the pool varies considerably between species (Sagisaka and Araki 1983, Rosnitschek-Schimmel 1985b, Sagisaka 1987). Those which preferentially accumulate arginine and proline, glutamate and glutamine, asparagine, or proline prior to overwintering have been reported (Sagisaka 1987). Fall increases in arginine occurred in leafy spurge, but increases in several other amino acids appeared to be more strongly associated with the overwintering process (Fig. 3). The largest increments occurred in asparagine, closely followed by those in glutamic acid, serine, ornithine, proline, arginine and aspartic acid.

Asparagine and glutamine, possessing low C to N ratios (4:2, 5:2), commonly are associated with transport and storage of nitrogen. The former is usually present in higher concentrations and is selectively catabolized in tissues requiring nitrogen and carbon for amino acid and protein synthesis (Sieciechowicz: *et al*. 1988). Asparagine appears to be the major amino acid for transportation of nitrogen remobilized during foliar senescence in leafy spurge (Fig. 3).

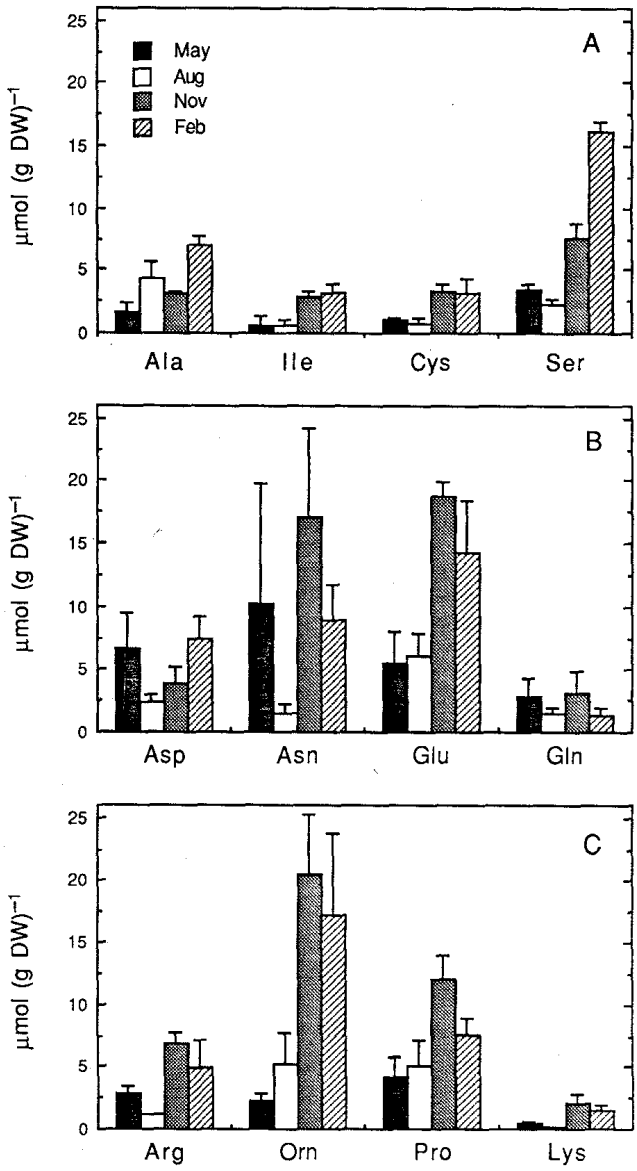

Fig. 3. (A) Alanine, cysteine, isoleucine and serine levels throughout the year in roots of leafy spurge. Data are means ± SE of 3 replicates, (B) Aspartic acid, asparagine, glutamic acid and glutamine levels throughout the year in roots of leafy spurge. Data are means ± SE of 3 replicates. (C) Arginine, ornithine, proline and lysine levels throughout the year in roots of leafy spurge. Data are means ± SE of 3 replicates.

Changes in the amino acid pool (Fig. 3) are indicative of other metabolic processes and adaptive roles. The apparent decrease in asparagine during February may reflect an interconversion to other amino acids, and the apparent increase in aspartic acid is suggestive of the deamidation of asparagine through the activity of amino transferases.

With the exception of chicory and dandelion (D. R. Cyr and J. D. Bewley, unpublished data), a seasonal flux of serine and ornithine (Fig. 3) has not been documented for roots of perennials. Serine possibly is functioning in its capacity as a carbon donor for biosynthetic reactions, while ornithine is a required intermediate for the cytosolic biosynthesis of arginine as well as for secondary products such as alkaloids and polyamines (Shargool *et al*. 1988). Proline, however, has often been associated with adaptation to various stress-associated phenomena (Paleg *et al*. 1981). Thus, the increases here may represent an important step in overwintering capacity. These different aspects of storage metabolism could be important targets for weed-control measures.

It appears that in the roots of most other herbaceous perennials, nitrogen is primarily partitioned into the free amino acid pool (Tromp 1983, Rosnitschek-Schimmel 1985a). Thus, it often has been assumed that proteins do not play significant roles in the storage of nitrogen in roots. However, water-extractable proteins are associated with cold (Koperzinskii 1939, Jung *et al*. 1967, Faw *et al*. 1976) and freezing tolerance (Perras and Sarhan 1989) of roots. The trends observed in soluble protein in leafy spurge are strongly suggestive of an important role in overwintering strategy. Changes in the soluble protein fraction resembled those of the free amino acid pool but the magnitude of fall-winter accumulations was significantly lower, and contents had declined prior to the resumption of activity in spring (Fig. 2). In it comprehensive survey of herbaceous perennials, Sagisaka (1987) did not observe fall accumulation of protein but did record significant reductions during spring. Thus, hydrolysis was associated with mobilization for new growth but not with winter changes in the free amino acid pool.

Nitrate + nitrite content of leafy spurge roots was at its lowest level during late summer (Fig. 2). A rapid increase in concentration during fall coincided with initial increments in amino acids and protein. However, levels remained high throughout the remainder of the year, in contrast to the transient accumulations recorded for chicory and dandelion (D. R. Cyr and J. D. Bewley, unpublished data). Although reduction of nitrate predominantly occurs in the cytosol, excess anion can readily accumulate in a vacuolar pool (Millard1988). In contrast, nitrite is rapidly reduced to ammonium in the plastid and does not accumulate significantly. Consequently, accumulation of nitrate + nitrite in leafy spurge is probably indicative of nitrate.Ammonium is readily incorporated into amino acids and proteins. Rosnitschek-Schimmel (1985c) showed stimulation of amide and arginine contents in response to exogenously supplied ammonium. Because assimilation of nitrogen appears to be limited by incorporation into amides (Oaks 1986), the major route for the assimilation of ammonia in roots, glutamine synthetase/glutamine-oxoglutarate aminotransferase (GS-GOGAT; A. Oaks and B. Hirel, cited in Millard 1988), is a pivotal point of regulation.

Lym and Messersmith (1987) have suggested that herbicide application should be timed to coincide with periods of increased translocation of total nonstructural carbohydrate (TNC to the roots and crowns of leafy spurge, or that a disruption of starch to sugar conversion would increase winter-kill. This approach may not be practical. Although overall seasonal patterns remained unaffected, they observed fluctuations in TNC levels that could not be explained on the basis of growth and developmental processes alone. In particular, levels of insoluble and soluble TNC were positively and inversely correlated with temperature, respectively. However, due to the presence of an extensive root system,

precipitation patterns had little effect on carbohydrate patterns. In view of these effects it may be difficult to optimize times of herbicide application or disruption of starch hydrolysis. The latter strategy might not prove effective in a single season due to the copious reserves of carbohydrates already present in roots. Nitrogenous compounds may represent a more effective metabolic target for control measures. The roles of important regulatory enzymes in nitrogen metabolism, i.e. GS-GOGAT, have not been studied extensively. A thorough understanding of these processes could aid in the development of more effective control measures for leafy spurge as well as for perennial weeds in general.

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