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PHOTOSYNTHATE PARTITIONING AND NITROGEN FIXATION
OF ALFALFA AND BIRDSFOOT TREFOIL

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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****

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1985

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I. Nodule activity and nitrogen concentration
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INTRODUCTION

Concern about dwindling supplies of fossil fuels has stimulated interest in the possibility of increasing the use and efficiency of biological nitrogen fixation for agricultural purposes. Much available literature indicates that there is a wide range in capacity of symbiotic nitrogen fixation in different legumes. An understanding of why some legumes fix more nitrogen than others will certainly provide information for the improvement of nitrogen fixation.

The capability of legume plants to fix nitrogen is dependent on the presence of the appropriate symbiotic Rhizobium species in the root nodules. Higher rates of nitrogen fixation by certain legumes may be due to factors associated with either the Rhizobium spp. or host plants. Much research aimed at increasing symbiotic nitrogen fixation of legumes has centered on the Rhizobium bacteria (Burton, 1972). Less information is available on plant factors which contribute to increasing nitrogen fixation capacity.
Legume hosts affect nitrogen fixation by one or more of the following factors. First, host genotype can influence effective nodule number and nodule mass per plant (Kossak and Bohlool, 1984) and these factors correlate with nitrogen fixation (Heichel, 1982). Second, the ability of the host plant to supply photosynthate to nodules has been shown to affect nitrogen fixation (Sheehy, Fishbeck, DeJong, Williams and Phillips, 1980). Third, utilization of photosynthate by nodules for nodule growth or for production of ATP and reducing power affect nitrogenase activity of nodules (Layzell, Rainbird, Atkins, and Pate, 1979).

Alfalfa has one of the highest rates of nitrogen fixation among legumes (Evans and Barber, 1977; Vance 1978; LaRue and Patterson, 1981). It has been calculated that alfalfa can fix up to 300 Kg nitrogen per hectare per year (Vance, 1978; LaRue and Patterson, 1981). Birdsfoot trefoil is also a temperate forage legume which is quite similar in growth habit and cultural requirements to alfalfa (Seaney and Henson, 1970). Relative to alfalfa, birdsfoot trefoil is a poor fixer of nitrogen (Cralle and Heichel, 1981). The overall purpose of the research described in this thesis is to examine some physiological and morphological characteristics which may be
contributing to this differential capacity for nitrogen fixation.

Birdsfoot trefoil roots generally have significantly more nodules and greater nodule mass than alfalfa (Vance, Johnson, Stade and Groat, 1982). Thus, it appears that factors other than nodule number and mass are responsible for the differential nitrogen fixation rates. Total herbage yield and net CO_2 assimilation rate are higher in alfalfa shoots than in birdsfoot trefoil shoots (Nelson and Smith, 1968; Cralle and Heichel, 1981). Thus, if photosynthetic availability is a limiting factor, alfalfa nodules might have the advantage of greater potential supply of photosynthate. In addition, total nonstructural carbohydrate (TNC) concentration in alfalfa roots (20 to 35% of total dry weight) has been shown to be higher than that of trefoil roots (10 to 15%) (Smith 1962; Nelson and Smith, 1968; Barta, 1973). This fact implies greater carbohydrate partitioning to alfalfa roots during growth. Hence, if alfalfa nodules are able to utilize root carbohydrates, they may be able to fix more nitrogen, especially during periods of low photosynthetic supply.

The significantly higher growth rate and net CO_2 assimilation of alfalfa compared to trefoil (Rhykerd, Langston, and Peterson, 1959; Nelson and Smith, 1968; Cralle and Heichel, 1981) suggest that differences in
photosynthate availability may be important. The significantly higher root TNC reserves maintained in alfalfa roots during active growth indicate that alfalfa roots are stronger sinks for photosynthate than are trefoil roots. In fact, it has been suggested that trefoil roots may experience carbohydrate deficiency during plant stress which results in their susceptibility to root and crown diseases (Barta, 1978). Because nodules are morphologically and physiologically different from roots, it is imperative that their ability to mobilize carbohydrate be examined apart from the total root system.

Recently Boller and Heichel (1983) suggested that alfalfa nodules are relatively weak sinks for current photosynthate compared with alfalfa roots. Their conclusion is tenuous, since nodules are reported by other researchers as strong sinks when compared to roots (Bach, Magee, and Burris, 1958; Small and Leonard, 1969; Streeter, 1980) or leaves (Sheikholeslam, Fishbeck, and Phillips, 1980). The fact that symbiotic nitrogen fixation in legumes requires significant carbon input (Neves, 1982) also contradicts Boller and Heichel's conclusion. Furthermore, alfalfa nodule sink strength was estimated by Boller and Heichel (1983) with $^{14}\text{C}\text{O}_2$ labeling using 24 h chase time. The $^{14}\text{C}$ recovered after 24 hours can not be considered as current photosynthate, since $^{14}\text{C}$ usually is
not redistributed after 24 h (Ryle, Powell, and Gordon, 1981a). Hence, there is a need to reevaluate the sink strength of alfalfa nodules.

Because there are such dramatic differences in photosynthate partitioning between alfalfa and trefoil, and because of the reported close relationship of nitrogen fixation to carbohydrate availability (Streeter, Mederski, and Ahmad, 1980; Caldwell, Fensom, Bordeleau, Thompson, Drouin, and Didsbury, 1984), it is desirable to carefully examine the pattern of photosynthate partitioning and metabolism in the nodule in these species. The objective of this study was to examine photosynthate partitioning within the plant and its metabolism in nodules. This was accomplished by first, characterizing photosynthate production and partitioning in the plant under normal environmental conditions. The plants were next subjected to environmental perturbations which upset normal photosynthate production and/or partitioning and subsequent effects on nitrogen fixation were measured. By measuring changes in nitrogen fixation rate in response to perturbations in carbohydrate supply and carbohydrate metabolism, it was hoped that more could be learned about the interaction of these factors in nitrogen fixation.

This study was initiated by measuring parameters which reflected the photosynthetic capacity of greenhouse
grown plants. By measuring CO₂ exchange rates (CER), starch accumulation patterns and related phenomena, one can characterize carbon fixation and partitioning in the leaves. If differential photosynthetic capacities are not found, then differences in nitrogen fixation between species may be less discernible.

Photosynthate partitioning and nitrogen fixation were studied using ¹⁴CO₂ labeling of shoots. Partitioning patterns were altered by shading and dark depletion studies. Dark starvation of nodules depletes available energy in nodules (Ching, Heddke, Russell, and Evans, 1975) so that upon return to light, recovery of nodule activity is closely associated with supply of photosynthate to nodules and their relative priority for available photosynthate (Sheehy et al., 1980).

Shading studies are also useful because as photosynthate becomes limiting, the relative sink strength of nodules should become more apparent. If a sink is deficient or marginally deficient under normal conditions, reduced light should yield greater response.

Increasing nodule size is correlated with the progression of nodule senescence and rate of nitrogen fixation (Vance et al., 1982). Specific nodule activity (SNA) and partitioning of ¹⁴C were compared among
different nodule size classes in order to learn more about the effective nodule mass in these species.

Because carbohydrate utilization and/or turnover rate in nodules may also be important in the differential response of these species, studies involving analysis of the distribution of $^{14}$C metabolites and respiration of nodules were also accomplished. Specific activity of metabolite pools is a useful indicator of metabolic rate and synthetic pathways, even though it is not definitive with regard to actual efficiency of carbohydrate utilization.

Further studies designed to examine capacity for nitrogen fixation centered on respiration activity and efficiency of alfalfa and trefoil nodules. If the potential metabolic activity of nodules is low in trefoil nodules, then improvement in photosynthate supply may have little importance in improving the fixation rate.
LITERATURE REVIEW

General characteristics of alfalfa and birdsfoot trefoil

The recognition of alfalfa (Medicago sativa L.) as the most popular forage legume can be ascribed to its high yield and nutritious forage. However, the use of alfalfa is not without its problems. The high protein content of alfalfa forage results in high bloat potential for ruminants grazing pure alfalfa pasture (Marten and Jordan, 1979). Alfalfa is very sensitive to soil acidity (Rhykerd and Overdahl, 1972), and is also highly susceptible to root rot diseases resulting from poor drainage and waterlogged soils (Barta, 1980).

Birdsfoot trefoil (Lotus corniculatus L.) is better adapted to those soils poorly adapted for alfalfa growth (Seaney and Henson, 1970; Barta, 1979). Birdsfoot trefoil also provides high quality forage which does not cause bloat (Marten and Jordan, 1979). In practice, it may be beneficial to substitute birdsfoot trefoil for alfalfa-grass in a pasture system (Marten and Jordan, 1979). However, the low yield potential of birdsfoot trefoil,
compared with alfalfa, is well documented (Yawalkar and Schmid, 1954; Nelson and Smith, 1969).

Crop yield might be described as a genetic program attuned to the environment. One important factor that affects yield is nitrogen input. Nitrogen is usually the rate limiting factor in crop yield (Barker and Mills, 1980). Since alfalfa and birdsfoot trefoil both obtain most of their nitrogen through symbiosis with *Rhizobium* spp., the amount of nitrogen fixed or the rate of nitrogen fixation is a major factor contributing to their yield (Burton, 1972). The nitrogen fixation rate of alfalfa, either measured on a whole plant or nodule dry weight bases, is higher than that of trefoil (Barta, 1978; Cralle and Heichel, 1981).

The greater shoot growth of alfalfa imposes a higher demand for nitrogen. This increased demand for nitrogen may stimulate greater nitrogen fixation which in turn increases the demand for more photosynthate. It is possible that nitrogen fixation limits growth, and the photosynthate available to the nodules limits nitrogen fixation.

**Rhizobia**

Microorganisms of the genus *Rhizobium* are aerobic, gram-negative, rod-shaped bacteria which occur naturally in many soils (Robertson and Farnden, 1980). The species
associated with alfalfa nodules is *Rhizobium meliloti*, while the species associated with birdsfoot trefoil is not well defined because of present taxonomic uncertainties. "Lotus rhizobia" is used to name the *Rhizobium* nodulating birdsfoot trefoil (Vincent, 1977).

*Rhizobium* assumes different sizes and shapes in the infected nodule cells. These altered *Rhizobium* cells are known as bacteroids. While bacteroids of birdsfoot trefoil appear only slightly larger than free-living bacteria (Vance et al., 1982), the bacteroids of alfalfa may increase their size five times or more than that of free-living bacteria (Miller and Tremblay, 1984).

Bacteroids are surrounded by a peribacteroid membrane of plant origin. There is only one bacteroid within the peribacteroid membrane in alfalfa nodules (Vance, Johnson, Halvorsen, Heichel, and Barnes, 1980). However, in mature nodules of birdsfoot trefoil, 12 to 16 bacteroids are enclosed in each peribacteroid membrane (Vance et al., 1982).

The shape and structure of the nodule

Nodules of different legumes have different shapes. Nodule shape is host plant determined and is usually consistent within genera. Nodules can be divided rather arbitrarily into three categories according to their
shapes, (a) elongate with indeterminate apical meristem and a basically cylindrical structure, (b) spherical nodules which have determinate meristem in several discrete meristem foci, and (c) collar nodules in which the meristematic activity becomes displaced to the lateral peripheries of the nodule so that the nodule grows around the root, as in lupins (Dart, 1975).

Nodules of alfalfa are elongate with the indeterminate meristem at the distal end (Hirsch, Long, Bang, Haskins, and Ausubel, 1982), and may be branched into comb shape (Bieberdorf, 1938). Spherical nodules of birdsfoot trefoil do not have a distinct meristematic zone and may have many discrete foci of meristematic activity in the nodule cortex (Vance et al., 1982).

It has been suggested that legume nodules of different shapes belong to different phylogenetic tribes of the family Leguminosae (Zhiznevskaya, I'lyasova, Troitskaya, Khailova, and Andreeva, 1979). Zhiznevskaya et al. (1979) concluded that the elongate nodules are found in phylogenetically younger legumes and have the highest nitrogen fixation activity per unit nodule weight. Spherical nodules, on the other hand, belong to the phylogenetically older legumes and have lower nitrogen fixation per unit nodule weight. Nutman (1956) also regarded the elongate nodule of alfalfa as the most
specialized and most highly developed form of symbiosis. However, the different growth patterns of the nodule meristem may contribute to their difference in nitrogen fixation activity. The spherical nodules with determinate meristem may have a large portion as the senescent zone, which can not fix nitrogen and reduces the amount of nitrogen fixed per unit weight.

The surface layer of elongate nodules of alfalfa is loosely packed with whitish parenchyma cells (Dixon, Blunden, and Searl, 1981), while the surface of birdsfoot trefoil nodule is corky and brownish, with ridges. These ridges are believed to be lenticels and may aid in gas exchange (Vance et al., 1982). The outermost surface of a nodule is usually made up of several layers of cortical cells. At the base of the nodule, one to five nodule vascular bundles may connect with the vascular system of the root. Nodule vascular traces of birdsfoot trefoil are located beneath the characteristic surface ridges (Pankhurst and Sprent, 1975).

Each nodule vascular trace has one or two layers of pericycle cells surrounding the phloem and xylem. For some nodules, these pericycle cells have dense cytoplasmic content and have developed into transfer cells. Transfer cells are believed not only to mediate the symplastic flow of sugars from the phloem to the infected tissue, but also
to secrete amino acids coming from bacteroids into the xylem (Dart, 1975). Transfer cells are most common in nodules having indeterminate meristem (Gunning, Pate, Minchin, and Marks, 1974). Pate, Gunning and Briarty (1969) observed transfer cells in nodules of alfalfa, but not in birdsfoot trefoil. They concluded that the existence of transfer cells increased the efficiency of nitrogen fixation.

Further inside the nodule cortical cells is a layer of sclereid cells separating the cortex and the central bacteroid zone. The sclereid cells have been suggested to be a barrier to the diffusion of water and gases, and may control the diffusion process in the nodule (Tjepkema and Yocum, 1974; Sinclair and Goudriaan, 1981). Most of the cells in the bacteroid zone are infected by rhizobia. Leghemoglobin which facilitates and regulates the flow of oxygen to the nitrogen fixing bacteroids, is present in the cytoplasm of these invaded cells (Robertson and Farnden, 1980). There is also some evidence that leghemoglobin is present at a lower concentration between the peribacteroid membrane and the rhizobia membrane (Bergersen and Appleby, 1981).

The nitrogen fixing activity of the nodule is located in the bacteroid zone due to the nitrogenase synthesized by the bacteroids. The bacteroid zone of birdsfoot
trefoil is in the central region of the nodule and may occupy 80 to 90% of the nodule cross sectional area. However, the bacteriod zone of a birdsfoot trefoil nodule is undifferentiated (Vance et al., 1982). Unlike birdsfoot trefoil, the bacteroid zone of alfalfa nodules, due to its indeterminate meristem, can be distinguished into meristem, early symbiotic zone, late symbiotic zone, and senescent zone (Hirsch et al., 1982).

Nodule senescence is closely related to nodule functioning. The senescent zone of a nodule usually has a green or brown color associated with the breakdown of leghemoglobin (Virtanen and Laine, 1946). Elongate nodules of alfalfa and spherical nodules of birdsfoot trefoil have different patterns of senescence. In alfalfa nodules with indeterminate apical meristem, senescence begins at the base of the nodule. The speed of senescence is dependent on the length of reduced photosynthate supply and stress conditions (Vance, Heichel, Barnes, Bryan and Johnson, 1979). The senescence of alfalfa nodules may occur concurrently with the new growth of symbiotic zone through the apical meristem of the nodule (Patel and Yang, 1980).

In nodules of birdsfoot trefoil, bacteroid zones are arranged concentrically with the oldest cells at the center of the nodule. When the nodule begins to senesce, tissues in the central region start to degenerate and the
senescent zone is enlarged toward the outer region (Tu 1975; Vance et al., 1982). Since the meristems of birdsfoot trefoil nodules cease to function after an initial growth, the initiation of new nodules in birdsfoot trefoil is necessary in order to provide new nitrogen fixing nodules.

The shorter persistence of birdsfoot trefoil nodules may affect its efficiency of nitrogen fixation, since much energy is needed for new nodule initiation. Alfalfa nodules may be more efficient in energy use, for the indeterminate meristem can provide new tissues for symbiosis without the need for new nodule formation.

Relationship of photosynthesis to nitrogen fixation

Nitrogen fixation through the symbiosis between the legume plant and Rhizobium spp. requires large quantities of energy. It has been estimated that 4 to 10 g of carbohydrates are used by nodules per g nitrogen reduced (Neves, 1982; Schubert and Wolk, 1982). Photosynthate supplied as carbohydrate and organic acid are used by nodules for (a) generation of reducing power and ATP to supply the nitrogenase system in the bacteroid, (b) maintenance of normal host cell cytosol metabolism, and (c) supply of carbon skeletons, ATP and reducing power for the
assimilation of ammonium into nitrogenous compounds (Rawsthorne, Minchin, Summerfield, Cookson, and Coombs, 1980).

Photosynthesis is the ultimate source of energy for biological nitrogen fixation. Many physiological factors that influence photosynthesis or photosynthate availability to nodules have a similar effect on nitrogen fixation. The various factors have been discussed by Silver and Hardy (1976), Streeter et al. (1980), and Havelka, Boyle, and Hardy (1982).

Increased light intensity not only increases the photosynthetic rate of the soybean, but also increases the specific activity (micromoles $\text{C}_2\text{H}_2$ reduced·g$^{-1}$ nodule dry weight) of nodules (Lawn and Brun, 1974). The specific activity of soybean nodules was also doubled after doubling the shoot/root ratio by grafting a second shoot to a single root system (Streeter, 1974). Carbon dioxide enrichment of field grown soybean plants resulted in increased shoot dry weight, seed yield, and nodule specific activity of soybeans (Hardy and Havelka, 1974). Within 5 hours after CO$_2$ enrichment an increase in the specific nitrogen fixation activity was observed in pea nodules, although long term CO$_2$ enrichment increased nitrogen fixation by increasing nodule mass (Phillips, Newell, Hassell, and Felling, 1976).
Increasing the photosynthetic rate by CO₂ enrichment has also been shown to increase alfalfa nodule specific activity (Sheehy et al., 1980). Bedmar and Olivares (1980) found an increase in specific nitrogen fixation activity of alfalfa nodules when photorespiration of leaves was inhibited by glyoxylate and isonicotinic acid hydrazide at concentrations of 300 and 100 mM, respectively. Removal of competitive sinks such as pods increased nitrogen fixation by presumably making more photosynthate available to soybean nodules (Ham, Lawn, and Brun, 1976).

Decreased light intensity by shading has been shown to reduce the specific activity of soybean nodules (Lawn and Brun, 1974). Defoliation of alfalfa and birdsfoot trefoil reduced nitrogen fixing activity of both species (Vance et al. 1979; Cralle and Heichel, 1981). Stem girdling to block the translocation of photosynthate decreased nodule activity immediately (Lawn and Brun, 1974; Wheeler, 1971). Water stress reduces photosynthetic rate, and also reduces nitrogen fixing activity of nodules (Sprent, 1972; Huang, Boyer, and Vanderhoef, 1975). The nitrogen fixation profiles are similar in soybean, peanut, and pea, with maximum nodule activity occurring in postanthesis followed by a sharp decline during the mid bean-filling stage when the reproductive sink demand was increased greatly (Hardy, Bruns, Hebert, Holsten, and
Photosynthate partitioning between the source and the sink

The distribution of photosynthate among various plant organs is of great importance for agricultural productivity. Photosynthesis and partitioning of photosynthate in relation to crop yield have been reviewed frequently (Watson, 1971; Hanson, 1979; Zeevaart, 1979; Gifford and Evans, 1981). Generally, it has been shown that there is no close relation between crop productivity and photosynthetic rate (Evans, 1975; Elmore, 1980; Gifford and Evans, 1981). This has led many authors to suggest that better control of photosynthate distribution to the usable parts of plant is more likely to increase yield. Zelitch (1982) argued that if seasonal canopy photosynthesis is used rather than the net CO$_2$ exchange per unit of leaf area, a good correlation can be found between photosynthesis and crop yield. However, he also agreed that the increased yield involved a greater allocation of photosynthate to the grain during the grain filling stage.

A source is defined as any plant part that has net export of carbon (Zeevaart, 1979). Source strength has been defined as the product of size and activity of the source (Wilson, 1972). Source strength can be readily
measured by the product of leaf area (source size) and net assimilation rate per unit leaf area (source activity). Hence, source strength is a function of leaf area, irradiance, CO₂ concentration, stomatal aperture, respiratory cost, and temperature (Zeevaart, 1979).

By the same token, a sink is any plant part that has a net import of carbohydrates. All actively growing, storing, or metabolizing tissues are sinks. Sinks may be meristematic, elongation, storage, or respiratory sinks (Evans, 1975).

Sink strength is also defined as the product of size and activity of the sink (Wilson, 1972). However, the size and activity of a sink may not be independent variables. Sinks are so dynamic that it may not be possible to apply a simple formula as described above. The size of a sink may be the major determinant of photosynthate partitioning. However, the shoot apex has very little size, a low relative growth rate and metabolic rate, yet, it continues to receive a supply of assimilates even if the plant is under stress (Evans, 1975). The shoot apex may have low sink strength according to the formula above, nevertheless it must be a sink of high priority.

Another measurement used to indicate sink strength is the rate of dry weight accumulation (Wareing and Patrick, 1975). However, dry weight accumulation data do not
account for respiration losses, and it also can not be used to measure the sink strength of the apical meristem. Nevertheless, dry weight accumulation provides some measure of the competitive ability of a sink. Wareing and Patrick (1975) suggested that the term 'sink strength' should refer to the potential capacity of a sink to accumulate photosynthate, whereas 'mobilizing ability' is applied to describe the resultant accumulation of dry matter by a sink within the competitive framework of a whole plant system.

**Photosynthate partitioning to the nodule**

By labeling source leaves with $^{14}\text{CO}_2$, considerable empirical knowledge about the pattern of photosynthate partitioning in the plant has been obtained. Photosynthate partitioning was found to be sensitive to the changes of source to sink ratio (Fellows, Egli, and Leggett, 1979), light intensity (Sheikholeslam et al., 1980), CO$_2$ concentration (Wardlaw, 1982), photoperiod (Chatterton and Silvius, 1980), temperature (Chatterton and Carlson, 1981), growth stage (Silvius, Kremer, and Lee, 1978), plant nutrition (Barta, 1975, 1976, and 1982), and air pollutants (McLaughlin and McConathy, 1983).

Photosynthate partitioning in legume plants in relation to nitrogen fixation has also been widely
investigated by the $^{14}$CO$_2$ labeling technique. Most $^{14}$CO$_2$ assimilated by leaves is accumulated in actively growing regions of the legume plant, and little is recovered in nodules (Latimore, Giddens, and Ashley, 1977; Boller and Heichel, 1983). Earlier studies of Bach et al. (1958) on soybeans found about 2.7 to 2.9% of $^{14}$C recovered in nodules with 19 and 10 hours of chase time, respectively. They showed that nodules of soybean are stronger sinks than roots by having 1.34 to 2.08 times higher specific radioactivity (dpm/g of dry weight).

Small and Leonard (1969) used three different chase times (1, 4, and 20 hours) to investigate photosynthate partitioning in response to combined N level in pea and clover. They found that clovers translocated photosynthate to nodules faster than peas. The recovery of $^{14}$C in nodules changed from 4.7 to 12.4% and 9.2 to 5.9%, using chase times of 4 to 20 hours, for pea and clover, respectively. They also concluded that supplying combined nitrogen decreased the proportion of photosynthate partitioned to nodules with a corresponding increase in the proportion going to roots.

Lawrie and Wheeler (1974) showed that $^{14}$C photosynthate recovered in pea nodules declined by 60% in response to flowering and fruit development, whereas net photosynthesis of the pea plant doubled. Apparently
strong sink demands of flowering and fruit development were able to modify photosynthate partitioning. Lawrie and Wheeler (1975) also found that the maximum $^{14}\text{C}$ recovered in bean (*Vicia faba* L.) nodules occurred after 90 min chase time and declined thereafter. From this observation, they concluded that photosynthate was rapidly metabolized on arrival in the nodule. They also found that the highest specific radioactivity in bean nodules, 90 min after labeling, was 3 times higher than the specific radioactivity of leaves or roots.

Using field grown soybeans, recovery of $^{14}\text{C}$ in nodules never exceeded 1% of total radioactivity recovered in the whole plant harvested 1 to 24 hours after labeling (Russell and Johnson, 1975). Latimore et al. (1977), however, recovered 3 to 6% of the $^{14}\text{C}$ in soybean nodules with a 3 hour chase time. Because their plants were grown in sand, greater recovery of nodules may be responsible for the greater $^{14}\text{C}$ recovery in nodules. However, Bach et al. (1958) found 2.7 to 2.9% recovery of $^{14}\text{C}$ in soybean nodules harvested from soil grown plants.

Sheikholeslam et al. (1980) found that pea plants grown under higher irradiance reduced more $\text{C}_2\text{H}_2$ and partitioned a greater portion of $^{14}\text{C}$ photosynthate to nodules. In the experiments of Ryle et al. (1981) where single clover leaves on the primary shoot were exposed to $^{14}\text{CO}_2$, 

nodulated plants generally exported more of their labeled assimilates to the nodulated roots than equivalent plants utilizing nitrate nitrogen.

There are four papers describing $^{14}$C recovered in nodules of alfalfa or birdsfoot trefoil. Barta (1979) presents only the specific radioactivity of birdsfoot trefoil nodules. Without total nodule dry weight, it is not possible to determine the total $^{14}$C recovered in nodules. However, his data showed that the specific radioactivity of birdsfoot trefoil nodules was less than shoots and roots. There was no effect of regrowth between 2 to 4 weeks after cutting on the specific radioactivity in birdsfoot trefoil nodules, although the nodule activity per plant had increased more than seven fold. Nodules of alfalfa contained only 0.2 to 0.6% of total $^{14}$C recovered by Barta (1982), with different levels of potassium treatment. Both alfalfa and birdsfoot trefoil plants in his experiments were sampled 12 days to 4 weeks after shoot removal. Although chase times varied from 1, 2.5 and 24 hours in his experiment, data of both species reported by Barta were considerably lower than nodules of other legumes discussed earlier.

Boller and Heichel (1983) and Vance, Heichel, and Barnes (1984) discussed $^{14}$C recovered in effective and ineffective nodules of alfalfa. They found the specific
radioactivity of effective nodules was higher than ineffective nodules. Their conclusion was that the capability of nitrogen fixation had no effect on partitioning of recent photosynthate to all organs except nodules, and that nodules of alfalfa are weak sinks. Their conclusion that alfalfa nodules are weak sinks may be tenuous, because the radioactivity recovered in nodules 24 hours after labeling represents residual $^{14}$C in the various organs. The $^{14}$C recovered was not the recent or current photosynthate, because $^{14}$C usually is not redistributed after 24 hours (Kouchi and Yoneyama, 1984).

Nodules use less than 20% of the photosynthate supplied to them for nodule growth (Neves, 1982). When the sink strength of nodules is compared with other organs, care should be taken. Nodules may have low 'mobilizing ability' for increasing their dry weight according to the definition of Wareing and Patrick (1975), but their 'sink strength' may be not correlated with their dry weight.

Both the recovery of $^{14}$C in nodules after various chase times and nodule dry weight fail to consider the loss of $^{14}$C by respiration. Respiration of nodules may account for more than 50% of total photosynthate input into nodules (Neves, 1982). Streeter et al. (1980) calculated that the consumption of carbohydrate by nodules appears to be two or three times as great as the theoretical energy
requirement for nitrogen fixation, hence there is an inefficient consumption of photosynthate in nodules. The evolution of hydrogen resulting from the wasteful reduction of protons (Schubert and Evans, 1977) and the presence of cyanide resistant respiration (Lambers, Layzell and Pate, 1980) are two possible mechanisms involved in this wasteful respiration.

The Australian group led by Pate has been working on carbon and nitrogen balance of various legumes. By carefully measuring the photosynthetic rate, respiration rate, and ¹⁴C partitioning, nodules of peas were estimated to use as much as one third of total photosynthate produced by the pea plant (Minchin and Pate, 1973), while lupin and cowpea nodules consumed 10 to 13% of the net photosynthate (Herridge and Pate, 1977).

The presence of phosphoenol pyruvate carboxylase in nodules complicates the estimation of the respiratory cost for nitrogen fixation. Vance, Stade, and Maxwell (1983) estimated that nodule CO₂ fixation by phosphoenol pyruvate carboxylase may provide 25% of the carbon required for assimilation of symbiotically fixed nitrogen in alfalfa nodules. Therefore, the measurement of CO₂ released by nodules must take into account production of CO₂, recapture of CO₂ by phosphoenol pyruvate carboxylase, or both.
At present, there is no good technique to calculate what proportion of photosynthate is allocated to nodules. The nodule as a sink is unique. It does not utilize a large amount of photosynthate for growth, but the nodule does 'process' a large amount of photosynthate. Since it is difficult to quantify the amount of photosynthate being partitioned to the nodule, the alternative is to use a combination of $^{14}$C partitioning, respiration, and nitrogen fixation data to estimate photosynthate partitioned to the nodule.

Utilization of $^{14}$C assimilates in the nodule

The majority of photosynthate translocated from the shoot to nodules is in the form of sucrose (Rawsthorne et al., 1980), hence the utilization of photosynthate may be viewed as carbohydrate metabolism. The neutral sugar fraction in nodules always contains the highest proportion of $^{14}$C within 24 hours after labeling. Amino acids are the product of ammonium assimilation and nitrogen fixation, therefore, they also contain high $^{14}$C. Bach et al. (1958) observed that organic acids in the soybean root had nearly twice the percentage of total $^{14}$C found in amino acids, whereas the amino acids in the nodule had almost twice the percent radioactivity compared to the organic acids.
In studying nodules of *Vicia faba*, Lawrie and Wheeler (1975) reported that the neutral sugar fraction contained more than 70% of the total radioactivity of ethanolic extracts between 0.5 to 6 hours after labeling. The nodule basic fraction, mostly amino acids, contained 10% of the 

\[ \text{recovered } 1 \text{.5 hours after labeling, but declined to 5% recovery 6 hours after labeling. Most of the radioactivity in the basic fraction was in glutamic acid, aspartic acid, glutamine, and asparagine. The nodule acidic fraction, mostly malic acid, accumulated no more than 2% of total recovered } \text{^14C in the ethanolic extracts.} \]

Reibach and Streeter (1983) reported that the \text{^14C} recovered in soybean nodules was maximum 3 hours after a 30 min labeling with \text{^14CO}_2. Bacteroids contained 10 to 30% of the total radioactivity 2 to 5 hours after labeling. Sixty to 65% of \text{^14C} in bacteroids was in the neutral sugar fraction. This \text{^14C} distribution suggested that the utilization of photosynthate for the generation of organic acids, mostly malate, and amino acids, mostly glutamate, was rapid.

**Cyanide-resistant respiration**

Cyanide-resistant respiration is widespread in higher plants and microorganisms (Meeuse 1975; Solomos, 1977). At a concentration of about 0.1 mM or lower,
cyanide is usually highly inhibitory to cytochrome oxidase (Knowles, 1976), while cyanide-resistant respiration contains an alternate oxidase which is insensitive to cyanide. The alternate oxidase of the cyanide-resistant pathway has not been purified and characterized. However, there is an agreement that the branch point of the cytochrome oxidase pathway and cyanide-resistant pathway in higher plants is at or near coenzyme Q (ubiquinone) prior to cytochrome b. Furthermore, the consensus product of cyanide-resistant pathway is water not H$_2$O$_2$. The cyanide-resistant pathway has also been shown to be a non-phosphorylating pathway (Laties, 1982).

Cyanide-resistant respiration in higher plants is inhibited by salicylhydroxamic acid (SHAM). The cyanide-resistant, SHAM-sensitive pathway has been shown to be involved in a variety of higher plant metabolic pathways. It is involved in thermogenic respiration (Meeuse, 1975), osmoregulation (Lambers, Blacquiere, and Stuiver, 1981), aging and senescence (Shingles, Arron, and Hill, 1982), flooding resistance (Carpenter and Mitchell, 1980), seed germination (Yu, Mitchell, Yentur, and Robitaille, 1979), stress metabolism (Alves, Heisler, Kissinger, Patterson, and Kalan, 1979) and regulation of carbohydrate supply (Lambers, 1982).
The energy overflow theory promoted by Lambers (1982) is of interest because it suggests that cyanide-resistant respiration may regulate photosynthate supply to roots and nodules. According to this theory, the alternate pathway is not coupled with ATP production, and is only engaged upon saturation of the cytochrome path. Saturation of the cytochrome path, the phosphorylating path, occurs when the activity of glycolysis and the TCA-cycle is so high that more NADH is generated than can be accommodated by the cytochrome path and biosynthesis reaction requiring NADH (Lambers and De Visser, 1984).

Lambers' group suggests that the alternate pathway is engaged at different levels in legume roots. Roots of legume-Rhizobium associations have a very low activity of the alternate pathway, whereas the roots of the same legume grown with inorganic nitrogen and without rhizobia have an operative alternate pathway (Lambers et al., 1980). Legume roots grown with inorganic nitrogen had 'wasteful' respiration. NADH is oxidized without phosphorylation to maintain the flux through the TCA-cycle and thus the production of precursors in biosynthetic processes under conditions of low ATP and NADH requirement (De Visser and Lambers, 1983).

Respiration of bacteroids represents most nodule respiration (Bergersen 1962). Although the basic
biochemistry of bacterial respiration is similar to higher plants (Haddock and Jones, 1977), there are many differences. Bacteria possess membrane-bound electron transport chains that in general terms are very similar to their counterpart in mitochondria of higher plants. However, the electron transport chain of bacteria shows variation in having different cytochromes. Bacteria also have versatile respiration via multiple electron transport chain.

Besides the phosphorylating components of the electron transfer chain, cytochrome b, c₁, c, and a–a₃, *Azotobacter* spp. have an additional cytochrome, cytochrome d, which is insensitive to rotenone and antimycin A, as well as a cyanide-resistant path (Barnes, 1973). The function of these two paths is believed to be respiratory protection for the oxygen sensitive nitrogenase, since *azotobacter* are aerobic nitrogen fixing bacteria. Cyanide sensitive respiration is more efficient in energy generation (Stouthamer, 1984), hence the overall efficiency of cellular energy conservation, phosphorylation, is low in *azotobacter* when dependent on atmospheric nitrogen (Haddock and Jones, 1977). Neijessel and Tempest (1976) termed similar protection in *Klebsiella*, also a nitrogen fixer, as an 'ATP-spill' reaction, which spill excess energy by ATP hydrolysis.
The electron transfer chain of *Rhizobium japonicum* includes cytochrome b and c, with cytochrome a-a$_3$ and cytochrome o as principal terminal oxidases (Appleby, Bergersen, Ching, Gibson, Gresshoff, and Trinick, 1981). Bacteroids inside legume nodules have a shift in the pattern of synthesis of cytochromes and other components of the electron transport chain, leading to the formation of a new respiratory chain that is functional under low O$_2$ concentration (Shanmugam, O'Gara, Anderson, and Valentine, 1978). Bacteroids of *Rhizobium japonicum* develop a unique cytochrome, P 450, instead of the cytochrome a-a$_3$ found in cultured cells (Appleby, 1969).

An ineffective terminal oxidase with apparent lower oxygen affinity was also observed in the bacteroidal respiratory system (Wittenberg, 1977). This ineffective oxidase is non-phosphorylating, therefore it wastes energy when electrons are passing through it. This ineffective terminal oxidase is believed to have a similar function as the cyanide-resistant path in azotobacter (Bergersen, 1984).
MATERIALS AND METHODS

Plant growth conditions

Seeds of 'Vernal' alfalfa and 'Viking' birdsfoot trefoil were grown in pots 20 cm in diameter and 23 cm deep containing silica sand. Before seeding, both alfalfa and birdsfoot trefoil seeds were inoculated with commercial strains of *Rhizobium meliloti* and 'Lotus rhizobia', respectively. Both inoculants were obtained from Agricultural Laboratories, Columbus, Ohio. Nodulation was excellent.

Each pot containing four plants was kept in a greenhouse compartment with a day/night temperature about 28 C/19 C. In addition to the natural sunlight in the greenhouse, metal halid lamps were used to provide approximately 300 to 500 μE·m⁻²·sec⁻¹ (300-700 nm) at canopy level and 14 hours of photoperiod. Plants were irrigated with nitrogen free nutrient solution daily. The nutrient solution contained nutrients in μmol l⁻¹: P, 197; K, 1292; S, 315; Mg, 399; Cl, 1280; Fe, 14.8; Ca, 104; Mn, 4.2; Cu, 0.2; Mo, 0.1; B, 1.0; and Zn, 0.1. Deionized water was
used to flush the pot once a week to avoid salt accumulation.

Plants used for experimentation were grown to early bloom to full bloom stages. This usually took two to two and a half months.

\(^{14}\text{C labeling procedure}\)

\(^{14}\text{CO}_2\) labeling was carried out in a sealed polyethylene bag. Approximately 15 (the time course experiment) or 25 \(\mu\text{Ci}\) (the dark and shading experiment) of \(^{14}\text{CO}_2\) were released into the bag by injecting 3 ml of 50% lactic acid into a 5 ml vial containing NaH\(^{14}\text{CO}_3\). Labeling was carried out between 1000 to 1100 hour for most of the studies. Plants were labeled for 30 min and then the bag was removed. Plants were harvested after a predetermined chase time. Sand was washed off the roots and roots were then frozen in liquid nitrogen within 3 min. The frozen plant tissues were freeze-dried and hand-separated into shoot, root, and nodule portions. The root and shoot tissues were ground through 40 mesh screen.

\textbf{Time course experiment}

Chase times of 1, 2, 3, 4, and 24 hours, were used to follow the \(^{14}\text{C}\) partitioning into different organs. The experimental design was a completely randomized design
with chase times as treatments which were replicated three times.

**Dark depletion experiment**

Plants were put into a dark growth chamber at 20 to 23°C for 40 hours. After return to the light, plants were labeled at 800 or 1300 hour, which were 0 or 5 hours after return to the light. The experimental design was a split plot design with treatments as the main plots and labeling times as the subplots.

**Shading experiment**

Plants were shaded for 7 days with 4 layers of cheese cloth to reduce the light intensity to approximately 20% of control plants at canopy level. The experimental design was a completely randomized design with three replications.

**Acetylene reduction assay**

The nodule activity was estimated using the acetylene reduction assay. Preliminary experiments showed that washing sand off root systems resulted in a 50% reduction of the acetylene reduction rate of alfalfa; therefore, in subsequent experiments, sand was shaken off the root. Decapitated plants were put into a 550 ml airtight Mason
jar fitted with a lid containing a septum to allow gas sampling with a syringe. Fifty ml of acetylene were injected into the jar through the septum, and the jar was then incubated at a room temperature (22 to 24°C). Gas samples of 1 ml were taken at 15 and 30 min after the start of incubation. These samples were assayed within two hours on a Hewlett Packard Model 402 gas chromatograph equipped with a flame ionization detector and alumina column. The carrier gas was helium (85 ml/min). Column temperature was 100°C, and the detector temperature was 105°C.

**Nitrogen concentration, chlorophyll concentration, and carbon exchange rate**

Nitrogen concentration of plant tissue was measured by the standard micro-Kjeldahl method.

Chlorophyll concentration was measured by the method of Arnon (1949). Fresh leaves (0.5 g) were ground in 15 ml of 80% (v:v) acetone. The extract was centrifuged at 2000 g for 15 min. Light absorption of the supernatant was measured at 663 and 645 nm with a Gilford spectrophotometer. Total chlorophyll concentration was calculated by the formula below:

\[
\text{Chlorophyll (mg/l)} = 20.2D_{645} + 8.02D_{663}.
\]

Where D is the absorbance at 645 nm or 663 nm.
The carbon exchange rate was measured in the laboratory. Leaves from the top to fifth leaf were enclosed in a leaf chamber. Artificial lighting, 800 μE m\(^{-2}\) sec\(^{-1}\) at leaf surface level, was filtered through a water bath to reduce heat transmission. Light intensity was measured with a Li-Cor radiometer, model LI-185B, Lambda Co.. Air flow rate was 3 l/min with a CO\(_2\) concentration about 335 ppm. The difference of CO\(_2\) concentration between the reference air flow and the flow through leaf chamber was determined with an infrared gas analyzer, Lira 200, MSA. The net carbon exchange rate (CER) was expressed as mg CO\(_2\) dm\(^{-2}\) h\(^{-1}\). The leaf area was measured with a Li-Cor portable area meter, model LI-3000, Lambda Co.,

**Specific leaf weight**

Specific leaf weight (SLW) was measured every 2 hours starting at 800 hour to 1800 hour. SLW was measured by taking 12 leaf discs from the top four to ten leaves with a hole puncher having a diameter of 0.64 cm. Leaf discs were freeze-dried and weighed.

**Soluble sugars and total nonstructural carbohydrates**

At the time of SLW sampling, leaf samples were also taken for total nonstructural carbohydrate (TNC)
measurement. Leaves were freeze-dried and ground to pass through 40 mesh screen. The extraction procedure for soluble sugars and TNC was basically the same as that of Barta (1978). Ground leaf tissues were shaken with water overnight. An insoluble pellet was separated by centrifugation at 1000 g for 15 min. The insoluble pellet was digested with 0.05% clarase (0.1 M acetate buffer, pH 4.45) for 40 hours. The soluble carbohydrates fraction and the clarase digested soluble fraction (starch) were assayed by the ferricyanide method (Hoffman, 1937) to obtain the reducing sugar concentration equivalent to the glucose standard. TNC was determined as the sum of the soluble sugar fraction and the starch fraction.

Extraction and fractionation of plant tissues

The procedure for extraction and fractionation are depicted in Fig. 1. Plant tissues, 100 mg roots or shoots, and 50 mg nodules, were soaked overnight in 15 ml methanol:chloroform:water mixture (MCW, 12:5:3, v:v:v). The tissues were extracted 3 times (15 ml x 3) with MCW. The supernatants were separated from the insoluble material by centrifugation at 1000 g for 15 min.

The MCW soluble supernatants were combined and evaporated to dryness at 40 C using a flash evaporator. The MCW soluble portions were redissolved in a
Fig. 1. Flow chart for recovery of $^{14}$C in tissue fractions.
GROUND TISSUE
40 mesh

soak overnight then
extracted 3X by
MeOH:CHCl₃:H₂O(12:5:3)

soluble

evaporate to
dryness

redissolve in
CHCl₃:H₂O(1:1)

water soluble

cation exchange
column (Dowex 50)

elute with
6N HCl

AMINO ACID
FRACTION

elute with
8N HCOOH

ORGANIC ACID
FRACTION

CHCl₃ FRACTION

H₂O fraction

insoluble

protein extraction
by pronase digestion

indigestible
by pronase

PROTEIN
FRACTION

starch extraction
by clarase digestion

indigestible
by clarase

STARCH
FRACTION

elute with
6N HCl

AMINO ACID
FRACTION

elute with
8N HCOOH

ORGANIC ACID
FRACTION

NEUTRAL SUGAR
FRACTION

RESIDUE
water:chloroform (1:1, v:v) solution. Chloroform soluble aliquots were mostly pigments. Water soluble aliquots were further separated by ion exchange columns as described by Splittstoesser (1969), Barta (1975 and 1976), and Dickson (1979). Water soluble aliquots were first allowed to pass through a Dowex 50 (X8, 100-200 mesh, H+ form) cation exchange column and then a Dowex 1 (X4, 100-200 mesh, formate form) anion exchange column. Those aliquots passing through both columns were collected as the neutral sugar fraction. Three volumes of deionized water were allowed to pass through both columns and eluted with the neutral fraction. The fraction retained by Dowex 50 was eluted by 5 volumes of 6 N HCl and collected as the amino acid fraction. And those retained by Dowex 1 were eluted by 8 N formic acid and collected as the organic acid fraction.

The MCW insoluble materials were first digested by pronase to obtain the protein fraction, and then digested by clarase to obtain the starch fraction. Those indigestible by pronase and clarase were considered as residue fraction. The pronase digestion was carried out by incubating MCW insoluble material with 20 ml pronase-buffer solution (0.02% pronase in 0.05 M Tris, pH 7.4) at 30 C for 24 hours. The remaining pellet after pronase digestion was digested by 0.1% clarase in 0.1 M acetate
buffer, pH 4.45, for 40 hours to obtain the starch fraction.

Radioactivity measurements

Aliquots of each fraction were dried in a scintillation vial using an air stream. Two ml of deionized water were added to redissolve the compounds in the vial before adding 8 ml of scintillation cocktail. Scintillation cocktail contained 24 g 2,5-diphenyloxazole (PPO) and 1 g p-bis(2(5-phenyloxazolyl)benzene) (POPOP) in 2 l toluene and 2 l Triton X-100. Radioactivities were measured using a scintillation counter and the standard channel ratio method was used for quench correction.

Respiration of nodules and bacteroids

Nodule respiration was measured polarographically with a YSI model 53 oxygen electrode (Yellow Springs, Ohio). Whole nodules or sliced nodules (1 mm in thickness) weighing 0.5 g were put into the YSI reaction vial containing 3 ml of 100 μM CaSO₄ (reaction solution). At least three minutes were allowed for the reaction solution to be saturated with atmospheric O₂ and equilibrated to the incubation temperature (24 C) by constant stirring. Respiration rates were determined by an O₂ electrode.
Bacteroids of alfalfa and birdsfoot trefoil were isolated by differential centrifugation. Nodules were ground in buffer contained 0.3M mannitol, 1.0mM EDTA, 10.0mM Tris, 2%(w/v) PVP, 0.1%(w/v) BSA, and 0.05%(w/v) cysteine, at pH 7.2. The washing buffer was the same as the grinding buffer except that the concentration of mannitol was 0.4M and no PVP or cysteine was added. The reaction buffer (pH 7.2) contained 0.3M mannitol, 10mM PO\textsubscript{4}\textsuperscript{2-}, 10mM KCl, and 5mM MgCl\textsubscript{2}.

Nodules (0.5g) were ground in a cold mortar with 5ml of grinding buffer, then filtered through 4 layers of cheese cloth. The mortar was rinsed with an additional 5ml of grinding buffer and the combined filtrates were centrifuged at 300 g for 10 min. The residue was discarded and the crude bacteroid fraction obtained by centrifugation at 5000 g for 10 min. The crude bacteroid pellet was washed with 5ml of washing buffer and recentrifuged at 5000 g for 10 min. The pellet was resuspended in 3 ml of reaction buffer.

The respiration rate of bacteroids was measured as described above. One ml of bacteroid suspension and 2 ml of reaction buffer were placed in a reaction vial. A magnetic stirrer was kept constantly rotating at the bottom of the reaction vial. At least 3 min were allowed for the saturation of O\textsubscript{2} and equilibration of the reaction vial.
temperature to that of the incubation chamber (24 °C) before inserting an oxygen electrode into the reaction vial.

Three respiratory inhibitors were investigated: KCN, SHAM (salicylhydroxamic acid), and disulfiram (tetraethylioperoxydicarbonic diamide). KCN and SHAM were dissolved in deionized water. Disulfiram was dissolved in absolute alcohol. Since SHAM also inhibits lipoxygenase activity, disulfiram which does not inhibited lipoxygenase activity was used to detect the possible interference from lipoxygenase. The lipoxygenase activity did not affect respiration rates appreciably as revealed by the disulfiram inhibition study; therefore, it was not included in later experiments.
RESULTS AND DISCUSSION

Photosynthesis and photosynthate partitioning in leaves

In order to investigate the potential photosynthate supply of alfalfa and trefoil shoots, chlorophyll concentration, net carbon exchange rate (CER), specific leaf weight (SLW), and carbohydrate concentration in leaves were measured. Chlorophyll concentration of alfalfa leaves was about twice as high as trefoil leaves (Table 1). CER of trefoil was only 78% of alfalfa leaves on a unit area basis (Table 1). Chlorophyll concentration was reported to increase linearly with CER in different alfalfa cultivars (Collins and Duke, 1981), however, extrapolation of this relationship between two species may not be justifiable. Alfalfa was reported to have significantly higher CO₂ fixation rate than trefoil (Rhykerd et al., 1959). Nelson and Smith (1969) reported that net assimilate rate of trefoil shoots was only 52 to 62% of alfalfa shoots. Alfalfa also had shoot dry weight (Appendix A, B, and C) and leaf area (Greub and Wedin, 1971; Cralle and Heichel, 1981) higher than trefoil of the same age. It is
Table 1. Chlorophyll concentration and CO$_2$ exchange rate of two month old alfalfa and birdsfoot trefoil leaves.

<table>
<thead>
<tr>
<th>Species</th>
<th>Chlorophyll concentration ($mg\cdot g^{-1}$ fresh weight)</th>
<th>CO$_2$ exchange rate ($mg CO_2\cdot h^{-1}\cdot dm^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>alfalfa</td>
<td>1.95</td>
<td>16.05</td>
</tr>
<tr>
<td>birdsfoot trefoil</td>
<td>1.07</td>
<td>12.49</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>0.18</td>
<td>2.83</td>
</tr>
</tbody>
</table>
reasonable to conclude that the total amount of photosynthate fixed by alfalfa shoots is greater than trefoil shoots.

The specific leaf weight (SLW), concentrations of soluble sugars, starch, and TNC of leaves are presented in Figure 2. All four parameters increased linearly from 800 to 1800 hours and had linear regression equations which were significantly different (p<.01) from each other except those of Fig. 2a which were not statistically different.

Figure 2a shows a similar pattern of SLW change for both species which suggests a similar dry weight accumulation rate in leaves of alfalfa and trefoil. However, Fig. 2b, 2c, and 2d show different patterns of carbohydrate accumulation. Alfalfa had higher concentrations of soluble sugar and TNC throughout all sampling times, however starch concentration in trefoil leaves was higher than alfalfa in early sampling times (Fig. 2c). Both starch and soluble sugars are available for extracellular translocation, but soluble sugars are more readily available for translocation (Herold, 1980). The greater soluble carbohydrate pool in alfalfa leaves could support a greater flux of photosynthate from shoots to roots and nodules.
Fig. 2. Relationship of alfalfa and birdsfoot trefoil specific leaf weight (SLW), leaf sugar, starch, and total nonstructural carbohydrate (TNC) concentration from 800 to 1800 hours.
a. SLW

\[ A = 2.24 + 0.18X, \quad r^2 = 0.53^{**} \]

\[ B = 3.33 + 0.11X, \quad r^2 = 0.37^{**} \]

A: alfalfa, △--△
B: birdsfoot trefoil, □--□

b. Sugar

\[ A = -3.26 + 0.98X, \quad r^2 = 0.88^{**} \]

\[ B = -3.32 + 0.79X, \quad r^2 = 0.92^{**} \]

c. Starch

\[ A = 3.25 + 0.22X, \quad r^2 = 0.66^{**} \]

\[ B = 4.86 + 0.14X, \quad r^2 = 0.76^{**} \]

d. TNC

\[ A = -0.01 + 1.20X, \quad r^2 = 0.90^{**} \]

\[ B = 1.54 + 0.92X, \quad r^2 = 0.94^{**} \]
Starch concentration usually has a diurnal cycle, reaching the peak at the end of the day and declining through the night to the lowest level at the beginning of day (Brown, Pearce, Wolf, and Blaser, 1972). Although starch concentrations of trefoil leaves might not be high enough to inhibit photosynthesis (Guinn and Mauney, 1980), a higher starch accumulation early in the day would result in relatively less photosynthate for extracellular translocation. Because trefoil had a lower chlorophyll concentration, lower CER, and higher starch concentration, early in the day, and all these factors influence photosynthate supply; the data suggest that photosynthates available for extracellular translocation from trefoil leaves are less than from alfalfa leaves.

Time course experiment

I. Nodule activity and nitrogen concentration of plant tissues

Dry weight of trefoil nodules was about 1.5 times greater than alfalfa nodules (Table 2). However, even with their greater nodule mass, trefoil had less nodule activity per pot than alfalfa (Table 2).

Specific nodule activity (SNA) is defined as mmol C₂H₂ reduced·h⁻¹·mg⁻¹ nodule dry weight. SNA of alfalfa
Table 2. Nodule activity and dry weight of alfalfa and birdsfoot trefoil. Plants were grown under the same conditions as the time course experiment. Nodule activities were determined by the acetylene reduction assay.

<table>
<thead>
<tr>
<th>Species</th>
<th>Acetylene reduction rate</th>
<th>Nodule dry weight per pot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol·h⁻¹·pot⁻¹</td>
<td>nmol·h⁻¹·mg⁻¹</td>
</tr>
<tr>
<td>alfalfa</td>
<td>19.60</td>
<td>169.25</td>
</tr>
<tr>
<td>birdsfoot</td>
<td>10.07</td>
<td>60.21</td>
</tr>
<tr>
<td>trefoil</td>
<td>9.15</td>
<td>40.25</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>9.15</td>
<td></td>
</tr>
</tbody>
</table>
(169.2 nmol h⁻¹ mg⁻¹ nodule dry weight) was 2.5 times greater than trefoil (60.2 nmol h⁻¹ mg⁻¹) (Table 2). The SNA of alfalfa nodules was reported to be the highest among eight legume species studied by Dart and Day (1971). Their results on alfalfa SNA were similar to data reported for alfalfa in Table 2. Maxwell, Vance, Heichel, and Stade (1984) also reported a 2.5 to 3-fold higher SNA in alfalfa, compared to trefoil.

Alfalfa had greater shoot and root dry weight than trefoil (Appendix A). Although alfalfa nodules comprised only 1.9% of the total plant dry weight, compared to 4.9% in trefoil nodules (Appendix A), nitrogen concentration of alfalfa shoots and roots was significantly higher than those of trefoil (Table 3) and were similar to that reported by Pierre and Banwart (1973). Since plants were grown in the absence of combined nitrogen, the product of dry weight and nitrogen concentration represents the total nitrogen fixed. The greater nitrogen fixed in alfalfa is consistent with the higher nodule activity of alfalfa nodules.

The consistently higher dry weight, nitrogen concentration, and total nodule activity of alfalfa plants and their lower nodule mass are the best evidence that alfalfa nodules are functioning more efficiently in
Table 3. Nitrogen concentration of alfalfa and birdsfoot trefoil shoots and roots expressed on a dry weight basis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Shoot</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>alfalfa</td>
<td>3.22</td>
<td>2.13</td>
</tr>
<tr>
<td>birdsfoot</td>
<td>3.00</td>
<td>1.83</td>
</tr>
<tr>
<td>trefoil</td>
<td>3.00</td>
<td>1.83</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>0.11</td>
<td>0.10</td>
</tr>
</tbody>
</table>
nitrogen fixation, based on unit nodule dry weight, compared to trefoil nodules. This is further supported by the higher SNA of alfalfa nodules. Higher nodule activity per unit alfalfa nodule weight may be due to several factors. First nodules with higher SNA may receive more photosynthate to support their greater energy needs. Secondly nodules with higher SNA may use available photosynthates more efficiently for nitrogen fixation.

II. $^{14}$C-photosynthate partitioning

The photosynthate partitioning pattern among different plant organs was demonstrated using the $^{14}$CO$_2$ labeling technique. Time course labeling experiments measured the $^{14}$C-photosynthate partitioning patterns 1, 2, 3, 4, and 24 hours after labeling.

The mean of the total radioactivity recovered per pot was $5.3 \times 10^6$ dpm. There was no significant difference in the total radioactivity recovered between alfalfa and trefoil and no significant difference among different chase times. Shoots of alfalfa and trefoil contained the highest percentage $^{14}$C radioactivity among the three plant parts (Fig. 3). The percentage $^{14}$C photosynthate in shoots of both species had a similar negative linear relationship with chase times from 1 to 4 hours after labeling (Fig.
Fig. 3. Relationship between chase time and percentage $^{14}$C recovered in alfalfa and birdsfoot trefoil shoots and roots. Two month old plants were pulse-labeled for 30 min and harvested after specific chase times.
Radioactivity (% of total)

- △ Alfalfa shoot
  $Y = 92.3 - 4.35X$, $r^2 = 0.83^*$

- □ Trefoil shoot
  $Y = 96.4 - 2.58X$, $r^2 = 0.57^*$

- ▲ Alfalfa root
  $Y = 5.73 + 4.06X$, $r^2 = 0.81^*$

- ■ Trefoil root
  $Y = 2.65 + 2.19X$, $r^2 = 0.57^*$

Chase Time (hour)
3). The decrease in percentage $^{14}$C in shoots suggests an export of $^{14}$C from shoots.

Alfalfa shoots consistently contained a lower percentage of the total $^{14}$C recovered for all chase times than trefoil shoots. Trefoil shoots retained 83% of the recovered $^{14}$C photosynthate 24 hours after labeling, while alfalfa shoots retained only 68% after 24 hours (Table 4). The lower percentage $^{14}$C recovered in alfalfa shoots indicates a higher proportion of $^{14}$C photosynthate being translocated out of shoots. Since the total amount of photosynthate fixed by alfalfa shoots was greater than trefoil shoots and alfalfa also exports a greater proportion of photosynthate out of shoots, the total amount of photosynthate exported by alfalfa shoots is higher than trefoil shoots. The higher percentage $^{14}$C retained by trefoil shoots also agrees with Barta's (1979) results indicating that trefoil shoot growth is the dominant sink for photosynthate.

The positive accumulation of $^{14}$C in roots of both species suggests that roots were the major sink receiving $^{14}$C photosynthates translocated out of shoots (Fig. 3). The similar values of the slopes in Fig. 3 indicate that the rate of $^{14}$C loss from shoots was about the same as the rate of $^{14}$C accumulation in the roots of each species.
Table 4. Percentage $^{14}$C recovered and specific radioactivity (SR) in alfalfa and birdsfoot shoots, roots, and nodules 24 h after labeling. Values are expressed as the mean of 3 replicates ± standard error.

<table>
<thead>
<tr>
<th>Organ</th>
<th>species</th>
<th>recovery</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% total</td>
<td>dpm·mg⁻¹ DW.</td>
</tr>
<tr>
<td>shoot</td>
<td>alfalfa</td>
<td>68.6±4.5</td>
<td>1904.5±521.2</td>
</tr>
<tr>
<td></td>
<td>trefoil</td>
<td>83.1±2.1</td>
<td>2738.9±385.7</td>
</tr>
<tr>
<td>root</td>
<td>alfalfa</td>
<td>29.7±4.5</td>
<td>700.2±143.1</td>
</tr>
<tr>
<td></td>
<td>trefoil</td>
<td>14.7±2.1</td>
<td>736.7±78.3</td>
</tr>
<tr>
<td>nodule</td>
<td>alfalfa</td>
<td>1.7±0.1</td>
<td>1161.8±192.6</td>
</tr>
<tr>
<td></td>
<td>trefoil</td>
<td>2.3±0.3</td>
<td>900.3±120.2</td>
</tr>
</tbody>
</table>
Alfalfa roots consistently had more than twice the percentage recovered $^{14}$C than roots of trefoil at all chase times (Fig 3 and Table 4). The higher percentage of $^{14}$C recovered in alfalfa roots suggests that alfalfa roots are stronger sinks than trefoil roots, and is consistent with the observation that alfalfa root TNC concentration is more than twice as great as TNC in trefoil roots (Smith, 1962; Nelson and Smith, 1969; Barta, 1978).

The percentage $^{14}$C recovered in nodules of alfalfa and trefoil was similar except that trefoil nodules had significantly lower $^{14}$C 1 hour after labeling (Table 5). The lower $^{14}$C recovery 1 hour after labeling suggests that the translocation of current photosynthates from shoots to nodules of trefoil was slower than that of alfalfa, since about the same percentage $^{14}$C was recovered in the nodule from 1 to 4 hours of chase times (Table 5).

The capacity of a sink to accumulate dry matter is defined by Wareing and Patrick (1975) as the 'mobilizing ability' of a sink within the competitive framework of a whole plant system. The higher proportion of radioactivity recovered in trefoil nodules compared to alfalfa nodules 24 h after labeling (Table 4) suggests that trefoil nodules had higher 'mobilizing ability' for dry matter accumulation. Furthermore, the lower dry weight of
Table 5. Percentage $^{14}$C recovered in nodules of alfalfa and birdsfoot trefoil in response to different chase times. Two month old plants were pulse-labeled for 30 min and harvested after specific chase time.

<table>
<thead>
<tr>
<th>Chase time (hour)</th>
<th>Alfalfa</th>
<th>Birdsfoot trefoil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% total</td>
<td>$^{14}$C recovered</td>
</tr>
<tr>
<td>1</td>
<td>2.03</td>
<td>0.94</td>
</tr>
<tr>
<td>2</td>
<td>2.85</td>
<td>2.16</td>
</tr>
<tr>
<td>3</td>
<td>2.89</td>
<td>2.50</td>
</tr>
<tr>
<td>4</td>
<td>2.99</td>
<td>2.10</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>N.S.</td>
<td>0.53</td>
</tr>
</tbody>
</table>
alfalfa nodules (Table 2) also indicates that alfalfa nodules had lower 'mobilizing ability' for dry matter accumulation. These two observations suggest that alfalfa nodules are using a lower proportion of photosynthates for nodule growth and function compared to trefoil nodules. Although Boller and Heichel (1983) suggested alfalfa nodules are weak sinks due to their low mass, caution must be exercised in interpreting the sink strength of nodules. It is not appropriate to compare nodule sink strength using 'mobilizing ability' and dry weight accumulation, because less than 20% of the photosynthate partitioned to nodules is used for nodule growth (Neves, 1982) and a greater proportion of photosynthate served as carbon skeleton for exporting nitrogenous compounds (Schubert and Wolk, 1982).

A better measure for comparison of sink strength among organs is the relative specific radioactivity (RSA) where the specific radioactivity (SR) (dpm·mg⁻¹) of each organ is compared with the mean SR of the whole plant (Boller and Heichel, 1983). The use of RSA can eliminate the effect of organ size on the percentage radioactivity recovered. Higher RSA represents stronger sink strength (Mor and Halevy, 1980). One of the most significant differences in the RSA data (Fig. 4), compared with the
Fig. 4. Relationship between chase time and relative specific radioactivity (RSA) of shoots, roots, and nodules of alfalfa and birdsfoot trefoil. RSA equals the specific radioactivity (SR) of each organ divided by the mean SR. The regression equations are: alfalfa shoot, \( Y = 1.96 - 0.06X \), \( r^2 = 0.09 \); trefoil shoot, \( Y = 1.61 - 0.04X \), \( r^2 = 0.33 \); alfalfa root, \( Y = 0.13 + 0.07X \), \( r^2 = 0.62 \); trefoil root, \( Y = 0.08 + 0.06X \), \( r^2 = 0.63 \); alfalfa nodule, \( Y = 0.83 + 0.22X \), \( r^2 = 0.51 \); trefoil nodule, \( Y = 0.19 + 0.08X \), \( r^2 = 0.54 \). "*": significant at 5% level. "***": significant at 1% level.
Relative Specific Activity (RSA)

A Alfalfa shoot
D — Trefoil shoot
A — Alfalfa nodule
B* • Trefoil nodule
A — Alfalfa root
— Trefoil root

Chase Time (hour)
data in Figure 3 and Table 5, is the high RSA value of alfalfa nodules, compared to alfalfa roots and trefoil nodules. Nodules may accumulate a lower proportion of the fixed $^{14}$C relative to roots (Table 5), however nodules of both species have higher radioactivity per unit weight, compared to roots.

Alfalfa nodules are extremely strong sinks. Alfalfa nodule RSA increased with chase times and approached a level similar to shoot RSA 4 hours after labeling (Fig. 4). The RSA data suggest that nodules of both species are stronger sinks than roots, and alfalfa nodules are stronger sinks than trefoil nodules. This is in contrast to the data of Boller and Heichel for alfalfa nodules (1983), but agrees with previous reports by Bach et al. (1958) and Lawrie and Wheeler (1975) for soybeans and Vicia faba, respectively. Boller and Heichel (1983) used a 24 h chase time to measure the sink strength for 'current photosynthate' which is not as closely related to 'current photosynthate' as the chase times used in this research (1 to 4, and 24 h). Even though the growth conditions and the experimental setup were different, alfalfa nodules relative to roots were found to be strong sinks for photosynthate because of their high SNA, RSA, and high respiration (Lamber and De Visser, 1984).
III. Photosynthate in the metabolic fractions

The relative accumulation and rate of change of $^{14}C$ photosynthate in various metabolite fractions may provide insight into the turnover of photosynthate. There were only slight variations of percentage $^{14}C$ recovered in the organic acid, amino acid, and chloroform fractions in shoots, roots, and nodules in response to chase times in both species. Sugar, starch, and residue fractions in alfalfa shoots were linearly correlated to chase times (Fig. 5), while only the trefoil shoot residue fraction responded to increasing chase time. The slopes of all regression equations were statistically different ($p<0.05$). The regression equations for sugar and starch activity in Fig. 5 seem to suggest that there was an interconversion of sugar to starch in shoots of both species, but in reality the increase in percentage $^{14}C$ of the starch fraction was due to the decline of total radioactivity in the shoot (Fig 3) while actual $^{14}C$ in the starch fraction remained relatively unchanged.

The proportion of radioactivity recovered in the residue fraction of trefoil shoots was higher than in alfalfa shoots over all chase times (Fig. 5 and Table 6). The proportion of $^{14}C$ photosynthates recovered in the residue fraction of trefoil shoots reached more than 3
Fig. 5. Relationship between chase times and percentage $^{14}$C recovered in the sugar, starch, and residue fractions of alfalfa and birdsfoot trefoil shoots. Two month old plants were pulse-labeled for 30 min and harvested after specific chase times.
- △ Alfalfa sugar
  \( Y = 49.4 - 5.49X, r^2 = 0.74 \)**
- □ Trefoil sugar
  \( Y = 36.9 - 3.29X, r^2 = 0.29 \)
- ▲ Alfalfa starch
  \( Y = 29.2 + 5.0X, r^2 = 0.64 \)**
- ■ Trefoil starch
  \( Y = 39.1 + 2.36X, r^2 = 0.24 \)
- ▲ Alfalfa residue
  \( Y = 1.98 + 0.55X, r^2 = 0.49 \)
- ■ Trefoil residue
  \( Y = 1.26 + 2.19X, r^2 = 0.72 \)**
fold that of alfalfa 24 h after labeling (Table 6). This result shows that trefoil shoots used a larger proportion of current photosynthates for structural growth, since the residue fraction represents the biosynthesis of proteins and cell wall polymers (Mor and Halevy, 1980).

The distribution of $^{14}$C-photosynthates in the sugar, starch, and residue fractions of roots in response to chase time was similar to the pattern observed in shoots of both species (Fig. 5, 6, and 7). While the percentage $^{14}$C in the sugar fraction of shoots was less than 45% from 1 to 4 hours (Fig. 5) after labeling, the sugar fraction in roots was always greater than 45% (Fig. 6). There appeared to be a true turnover of sugar to the starch and residue fractions in roots (Fig. 6 and 7), since total radioactivity was rising in the roots (Fig. 3). The greater turnover of the sugar fraction in trefoil roots suggests a smaller pool of soluble sugars, hence the incoming sugars from trefoil shoots were used more rapidly. The higher proportion of $^{14}$C recovered in the organic acid fraction of trefoil roots 1 to 4 (data not shown) and 24 h (Table 6) after labeling also agrees with the hypothesis that incoming sugars are used rapidly by trefoil roots, since organic acids are the immediate products of respiration. This phenomenon might be predicted because
Table 6. Percentage $^{14}$C recovered in the metabolic fractions of shoots, roots, and nodules 24 h after labeling. Values are expressed as mean of 3 replicates ± standard error.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sugar</th>
<th>Organic acid</th>
<th>Amino acid</th>
<th>Starch</th>
<th>Chloroform</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alfalfa</td>
<td>17.8 ± 4.8</td>
<td>6.5 ± 1.3</td>
<td>4.0 ± 0.9</td>
<td>59.6 ± 7.8</td>
<td>6.2 ± 1.1</td>
<td>6.0 ± 0.8</td>
</tr>
<tr>
<td>trefoil</td>
<td>14.7 ± 1.8</td>
<td>7.0 ± 0.5</td>
<td>3.2 ± 0.2</td>
<td>48.4 ± 1.0</td>
<td>7.1 ± 0.2</td>
<td>19.7 ± 1.1</td>
</tr>
<tr>
<td>Root</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alfalfa</td>
<td>62.6 ± 6.3</td>
<td>4.3 ± 0.6</td>
<td>9.4 ± 1.2</td>
<td>20.3 ± 3.9</td>
<td>1.3 ± 0.5</td>
<td>2.1 ± 1.6</td>
</tr>
<tr>
<td>trefoil</td>
<td>47.8 ± 10.9</td>
<td>11.9 ± 1.8</td>
<td>8.9 ± 2.2</td>
<td>21.4 ± 7.3</td>
<td>3.7 ± 0.7</td>
<td>6.4 ± 0.9</td>
</tr>
<tr>
<td>Nodule</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alfalfa</td>
<td>50.9 ± 9.0</td>
<td>7.1 ± 1.1</td>
<td>17.0 ± 2.7</td>
<td>16.7 ± 2.9</td>
<td>3.4 ± 1.0</td>
<td>5.0 ± 1.5</td>
</tr>
<tr>
<td>trefoil</td>
<td>38.1 ± 5.2</td>
<td>8.7 ± 0.6</td>
<td>25.5 ± 3.2</td>
<td>12.7 ± 0.5</td>
<td>3.5 ± 0.3</td>
<td>11.6 ± 1.6</td>
</tr>
</tbody>
</table>
Fig. 6. Relationship between chase time and percentage $^{14}$C recovered in the sugar fraction of alfalfa and birdsfoot trefoil roots. Two month old plants were pulse-labeled for 30 min and harvested after specific chase times.
Alfalfa sugar
\[ Y = 87.4 - 2.14X, \quad r^2 = 0.34^* \]

Trefoil sugar
\[ Y = 88.5 - 5.67X, \quad r^2 = 0.63^{**} \]
Fig. 7. Relationship between chase time and percentage $^{14}$C recovered in the starch and residue fractions of alfalfa and birdsfoot trefoil roots. Two month old plants were pulse-labeled for 30 min and harvested after specific chase times.
Trefoil starch: $Y = 0.66 + 2.57X$, $r^2 = 0.77$

Alfalfa starch: $Y = 1.59 + 1.27X$, $r^2 = 0.33$

Trefoil residue: $Y = -0.18 + 0.68X$, $r^2 = 0.69$

Alfalfa residue: $Y = 0.23 + 0.06X$, $r^2 = 0.33$
the concentration of total nonstructural carbohydrates in trefoil roots is very low, compared to alfalfa roots (Nelson and Smith, 1968; Barta, 1978; Cralle and Heichel, 1981).

The pattern of $^{14}$C starch accumulation in roots was similar to the pattern observed in shoots. The $^{14}$C accumulation in the residue fraction of trefoil roots was higher than in alfalfa roots at all chase times (Fig. 7). However, the amount of total photosynthate found in the trefoil root residue fraction may not necessarily be higher than alfalfa roots, because the proportion of photosynthate exported from the shoots to the roots was greater in alfalfa than in trefoil (Fig. 3).

The pattern of $^{14}$C distribution among the sugar, starch and residue fractions in nodules (Fig. 8 and 9) was similar to that found in roots (Fig. 6 and 7). Both roots and nodules accumulated a low percentage $^{14}$C in the starch fraction for the first 4 hours after labeling, but this percentage accumulation increased 3 fold 24 hours after labeling for roots and nodules of both species (Table 6, Fig. 7, and 9). Trefoil roots and nodules always contained a higher percentage $^{14}$C in the starch fraction than alfalfa roots and nodules. Since starch storage has been suggested to compete with nodules for photosynthate
Fig. 8. Relationship between chase time and percentage $^{14}$C recovered in the sugar fraction of alfalfa and birdsfoot trefoil nodules. Two month old plants were pulse-labeled for 30 min and harvested after specific chase times.
Radioactivity (\% \textsuperscript{14}C recovered in the nodule)

- Alfalfa sugar
  \[ Y = 85.8 - 4.46X, \quad r^2 = 0.63^{**} \]

- Trefoil sugar
  \[ Y = 76.0 - 6.29X, \quad r^2 = 0.35^{*} \]
Fig. 9. Relationship between chase time and percentage $^{14}\text{C}$ recovered in starch and residue fractions of alfalfa and birdsfoot trefoil nodules. Two months old plants were pulse-labeled for 30 min and harvested after specific chase chase times.
Radioactivity (% $^{14}\text{C}$ recovered in the nodule)

- **Alfalfa starch**: $Y = -0.58 + 1.07X$, $r^2 = 0.89**$
- **Trefoil starch**: $Y = -0.34 + 1.42X$, $r^2 = 0.76**$
- **Alfalfa residue**: $Y = -0.06 + 0.25X$, $r^2 = 0.76**$
- **Trefoil residue**: $Y = -0.05 + 1.23X$, $r^2 = 0.59**$

Chase Time (hour)
(Sheehy et al., 1980). The greater accumulation of $^{14}$C in the starch fraction of trefoil shoots, roots, and nodules may compete with nitrogen fixation for current photosynthate.

Nodules, since they are the site of nitrogen fixation and ammonium assimilation, contained a much higher percentage of fixed $^{14}$C in the amino acid fraction than shoots (Table 6). Roots had an intermediate percentage $^{14}$C in the amino acid fraction (Table 6). This result agrees with the study on *Vicia faba* by Lawrie and Wheeler (1975). The percentage $^{14}$C recovered in the chloroform and residue fractions of trefoil nodules were always higher than those of alfalfa nodules (Table 6), while the percentage $^{14}$C in the sugar fraction of alfalfa nodules was higher than that in trefoil nodules (Fig. 8).

The three most active metabolic fractions in nodules are sugars, organic acids and amino acids, and have been studied more frequently (Lawrie and Wheeler, 1975; Reibach and Streeter, 1983). The pattern of radioactivity distribution in alfalfa and trefoil nodules (Fig. 10) is basically similar to pattern in nodules of *Vicia faba* (Lawrie and Wheeler, 1975) and soybean (Reibach and Streeter, 1983). The maximum of total radioactivity was recovered 2 h after labeling in alfalfa, trefoil (Fig.
Fig. 10. Specific radioactivity (SR) of total, neutral sugars, organic acids, and amino acids in alfalfa and birdsfoot trefoil nodules. Vertical bars indicate two standard errors (S.E.). Where the vertical bars do not cross the symbol indicate only one S.E. and no vertical bar indicates that S.E. is smaller than the symbol.
10), and *Vicia faba* (Lawrie and Wheeler, 1975) nodules, while soybean nodules peaked 3 h after labeling (Reibach and Streeter, 1983). The higher sink strength of alfalfa nodules is also shown by the greater amount of total radioactivity recovered per nodule unit dry weight (Fig. 10).

The specific radioactivity (SR) of the sugar fraction (Fig. 10) represented the highest proportion of radioactivity (Fig. 8 and 9) recovered in nodules and followed a similar change as in the total radioactivity recovered per mg nodule dry weight (dpm·mg⁻¹) (Fig. 10). There was an increase of radioactivity recovered in the organic acid and amino acid fractions from 1 to 2 h chase time (Fig. 10). These data further suggest the observation that there is a rapid turnover of sugars to organic acids and amino acids in the nodules (Lawrie and Wheeler, 1975; Reibach and Streeter, 1983).

So far there have been two consistent differences between shoots, roots, and nodules of alfalfa and trefoil in the partitioning of ¹⁴C-photosynthate. First, alfalfa contained a higher proportion of ¹⁴C in the sugar fraction of all plant parts than trefoil. The higher percentage in the sugar fraction could influence the amount of current photosynthates of alfalfa in the readily available form
due to the higher carbon fixation rate of alfalfa shoots (Table 1). More rapid synthesis of starch in trefoil may explain the lower percentage $^{14}\text{C}$ in the sugar fraction of trefoil shoots.

The second consistent difference was the higher percentage $^{14}\text{C}$ recovered in the residue fractions of trefoil shoots, roots, and nodules. This indicates that trefoil uses a higher proportion of current photosynthates for growth. The relatively higher rate of nitrogen fixation in cowpea (*Vigna unguiculata* L.) nodules, compared with lupin (*Lupinus albus* L.) nodules, has been suggested to be due in part to a smaller allocation of photosynthates to nodule growth (Layzell, Rainbird, Atkins, and Pate, 1979). Since alfalfa nodules used a lower proportion of $^{14}\text{C}$ photosynthate for nodule growth, a greater amount of $^{14}\text{C}$ photosynthate may be available for nitrogen fixation.

**Dark depletion experiment**

I. Nodule activity and nitrogen concentration

Carbohydrate reserves in legume plants can be used to support nodule activities at night. Placing plants in the dark for a period of 40 hours should reduce the available carbohydrates and nitrogen fixation (Ching, Hedtke, Russell, and Evans, 1975). At both sampling times, 0 and
5 hours after the plants were returned to the light, nodule activity, expressed on a whole pot basis, and specific nodule activity of both species were significantly reduced as a result of the dark treatment (Table 7), except for nodule activity of trefoil at 0 hour (whole pot basis). Sheehy et al. (1980) reported an 80% reduction of alfalfa nodule activity caused by 40 hours of dark depletion. A similar reduction in nodule activity was also reported in dark treated pea plants by Virtanen et al. (1955). These observations agree with reports that energy supply and carbohydrate level in dark treated nodules were significant reduced (Ching et al., 1975; Sheehy et al., 1980).

Both the total nodule activity and nodule dry weight of control plants used in the dark experiments (Table 7) were higher than similar data reported for control plants used in the time course experiment (Table 2). Although plants used in both experiments were greenhouse grown plants of the same age, plants used in the dark experiment were grown in late spring. The better ambient light condition probably provided better plant growth and higher carbohydrate concentration in plant tissues than plants grown in early spring for the time course experiment.
Table 7. Alfalfa and birdsfoot trefoil nodule activity and nodule dry weight after 40 h of dark. There were two measurements which were 0 and 5 h after return to light, respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acetylene reduction rate</th>
<th>Nodule dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu M \cdot h^{-1} \cdot pot^{-1} )</td>
<td>( nM \cdot h^{-1} \cdot mg^{-1} )</td>
</tr>
<tr>
<td>0 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alfalfa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>50.0</td>
<td>204.5</td>
</tr>
<tr>
<td>dark</td>
<td>8.5</td>
<td>46.4</td>
</tr>
<tr>
<td>birdsfoot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>42.3</td>
<td>72.7</td>
</tr>
<tr>
<td>dark</td>
<td>15.2</td>
<td>27.6</td>
</tr>
<tr>
<td>5 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alfalfa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>77.9</td>
<td>309.0</td>
</tr>
<tr>
<td>dark</td>
<td>27.1</td>
<td>117.5</td>
</tr>
<tr>
<td>birdsfoot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>73.0</td>
<td>103.0</td>
</tr>
<tr>
<td>dark</td>
<td>20.9</td>
<td>42.3</td>
</tr>
<tr>
<td>LSD(0.05)</td>
<td>27.4</td>
<td>76.2</td>
</tr>
</tbody>
</table>
Although total nodule activity was affected by season, the relative differences in specific nodule activities and nodule dry weights between alfalfa and trefoil in control plants were consistent. The specific nodule activity of alfalfa was about 3 times higher than trefoil, while nodule dry weight of trefoil was two to three times higher than alfalfa (Table 2 and 7).

Nitrogen concentrations in alfalfa shoots and roots were both higher than those in trefoil (Table 8), differences which corresponded to the nitrogen fixation of the species in the time course experiment (Table 3). Nitrogen concentrations of 40 hours dark treated plants were significantly higher than control plants. This suggests that plant carbohydrates were consumed during the dark treatment (Sheehy et al., 1980), but plant nitrogen remained unchanged.

II. $^{14}$C-photosynthate partitioning

Since nodule specific radioactivity of the time course experiment reached a maximum 2 hours after labeling (Fig. 10), the two hours chase time was used in all treatments in the dark experiment. The distribution of $^{14}$C among shoots, roots, and nodules at 0 hour and 5 hours after return to the light were not statistically
Table 8. Nitrogen concentration of alfalfa and birdsfoot trefoil shoots and roots after 40 h dark depletion.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrogen concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of dry weight</td>
</tr>
<tr>
<td></td>
<td>alfalfa</td>
</tr>
<tr>
<td>shoot</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>2.45</td>
</tr>
<tr>
<td>dark</td>
<td>2.90</td>
</tr>
<tr>
<td>root</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>1.95</td>
</tr>
<tr>
<td>dark</td>
<td>2.17</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>0.22</td>
</tr>
</tbody>
</table>
different, hence, the data were pooled and are presented in Table 9.

The mean radioactivity recovered per pot was $11.2 \times 10^6$ dpm. There was no significant difference in radioactivity recovered between species or treatments. With dark treatment for 40 hours, both species retained a greater proportion of the total photosynthate in the shoots after return to the light (Table 9). The reduction in photosynthate translocation out of shoots suggests that shoot carbohydrate pools were diminished making the shoot a more competitive sink. Pearce, Fissel, and Carlson (1969) reported that the reduction of photosynthate by defoliation also stimulated alfalfa shoot regrowth to become the dominant sink for photosynthate.

The dark treatment significantly reduced photosynthate translocation to roots and nodules of both species (Table 9). Compared to the control, the reduction in $^{14}$C recovered in nodules was 87 to 92%, for trefoil and alfalfa nodules, respectively; while the reduction in $^{14}$C accumulation in roots was less severe (67 to 78%). This suggests that roots and nodules are less competitive than the shoots, and nodules are less competitive than the roots for photosynthate with 40 h of dark depletion.
Table 9. Percentage \(^{14}\text{C}\) recovered from alfalfa and birdsfoot trefoil shoots, roots and nodules after 40 h dark depletion. Two month old plants were pulse-labeled for 30 min and harvested 2 h after labeling.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot</th>
<th>Root</th>
<th>Nodule</th>
<th>% total (^{14}\text{C}) recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>alfalfa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>82.07</td>
<td>14.06</td>
<td>3.38</td>
<td></td>
</tr>
<tr>
<td>dark</td>
<td>96.62</td>
<td>3.06</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>4.83</td>
<td>3.88</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td><strong>birdsfoot trefoil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>84.27</td>
<td>11.31</td>
<td>4.41</td>
<td></td>
</tr>
<tr>
<td>dark</td>
<td>95.64</td>
<td>3.77</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>3.64</td>
<td>3.39</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>
Greater sink strength as expressed by RSA (Table 10) was observed in nodules relative to roots of both species. This is consistent with previous data (Fig. 4) and other reports (Bach et al., 1958; Sheikholeslam et al., 1980). Dark treated shoots of both species have significantly higher RSA than control shoots while RSA of dark treated roots and nodules were reduced significantly except for trefoil nodules (Table 10). Shoot sink strength in both species was increased by 40 hours of darkness and is consistent with the earlier discussion that the shoot becomes a more dominant sink after 40 h of darkness (Table 9). Forty hours of darkness resulted in a decline in the nodule activity (Table 7) and the relative sink strength of roots and nodules (Table 9 and 10). The magnitude of reduction by dark in nodule RSA of both species was more severe than in root RSA, even though nodules still remained a RSA similar to roots (Table 10). The data suggest that nodules are strong sinks under normal light condition (Fig. 4 and Table 10), but nodules can not compete effectively after plant carbohydrate depletion caused by 40 hours dark.

Although there was no difference in $^{14}$C-photosynthate partitioning among plant organs between 0 and 5 hours after return to the light, the partitioning patterns among
Table 10. Relative specific radioactivity (RSA) in alfalfa and birdsfoot trefoil shoots, roots, and nodules after 40 h of dark. RSA equals the specific radioactivity (SR) of an organ divided by the mean SR of the whole plant.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot</th>
<th>Root</th>
<th>Nodule</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RSA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alfalfa control</td>
<td>1.57</td>
<td>0.31</td>
<td>1.74</td>
</tr>
<tr>
<td>dark</td>
<td>1.81</td>
<td>0.07</td>
<td>0.16</td>
</tr>
<tr>
<td>birdsfoot trefoil</td>
<td>1.34</td>
<td>0.35</td>
<td>0.79</td>
</tr>
<tr>
<td>dark</td>
<td>1.59</td>
<td>0.11</td>
<td>0.12</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>0.15</td>
<td>0.12</td>
<td>0.78</td>
</tr>
</tbody>
</table>
seven nodule metabolite fractions were significantly affected by the length of time after return to the light. Two fractions, in particular sugar and amino acid, were significantly affected by dark treatment (Table 11). At 0 hour after return to the light, alfalfa and trefoil nodules contained significantly less $^{14}$C in the sugar fraction and more $^{14}$C in the amino acid fraction (Table 11). This indicates a more rapid metabolism of sugars to amino acids, compared to the control.

The percentage $^{14}$C recovered in the amino acid fraction of dark treated alfalfa nodules was extraordinary in that about 80% of the $^{14}$C in alfalfa nodules was in the amino acid fraction, compared with only about 42% of trefoil nodules (Table 11). The rapid turnover of $^{14}$C photosynthate in alfalfa nodules suggests more active metabolism possibly due to the higher rate of nitrogen fixation.

Although nodule activity of dark treated plants had not returned to the control level 5 hours after return to the light (Table 7), dark treated nodules displayed a normal $^{14}$C photosynthate partitioning pattern 5 hours after return to the light (Table 11). The significance of this different response in nodule activity and metabolite distribution is unknown.
Table 11. Percentage $^{14}$C recovered from nodules collected from plants after 40 h dark depletion. Nodules were harvested 2 h after a 30 min pulse-labeling. There were two measurements, 0 and 5 h after return to light.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sugar</th>
<th>Organic amino acid</th>
<th>Starch</th>
<th>Protein</th>
<th>Chloro-Residue form</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% $^{14}$C recovered in the nodule</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alfalfa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>64.98a*</td>
<td>1.55</td>
<td>21.13b</td>
<td>7.07</td>
<td>1.87</td>
</tr>
<tr>
<td>dark</td>
<td>9.44b</td>
<td>1.57</td>
<td>79.29a</td>
<td>4.97</td>
<td>1.94</td>
</tr>
<tr>
<td>5 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>65.39a</td>
<td>1.98</td>
<td>15.57b</td>
<td>3.70</td>
<td>4.09</td>
</tr>
<tr>
<td>dark</td>
<td>77.19a</td>
<td>1.98</td>
<td>13.58b</td>
<td>3.90</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>birdsfoot trefoil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>71.19ab</td>
<td>1.77</td>
<td>15.95b</td>
<td>5.84</td>
<td>3.08</td>
</tr>
<tr>
<td>dark</td>
<td>51.63b</td>
<td>1.75</td>
<td>41.88a</td>
<td>2.51</td>
<td>0.73</td>
</tr>
<tr>
<td>5 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>61.67ab</td>
<td>1.91</td>
<td>22.76ab</td>
<td>6.47</td>
<td>3.48</td>
</tr>
<tr>
<td>dark</td>
<td>78.32a</td>
<td>2.54</td>
<td>9.06b</td>
<td>3.76</td>
<td>2.95</td>
</tr>
</tbody>
</table>

*means followed by the same letter in the same column within the same species are not statistically different at 5% level.
Shading experiment

I. Nodule activity and nodule dry weight

Seven days of 80% shading had an effect on nodule activity similar to the effect noted with the 40 hours dark treatment (Table 12 and 7). This is consistent with the effect of shading on nodule activity of soybeans (Lawn and Brun, 1974), Phaseolus vulgaris (Antoniw and Sprent, 1978), and peas (Sheikholeslam et al., 1980). Both total nodule and specific nodule activities were significantly reduced by shading in both species studied (Table 12). The lower specific nodule activity of shaded nodules observed in this study suggests that nodules under shade are receiving a lower amount of photosynthate or there is lower demand for nitrogen.

The magnitude of reduction in nodule activity was greater in trefoil than in alfalfa (Table 12). The data in Table 12 appear to agree with other reports that shading has a more adverse effect on trefoil than alfalfa (McKee, 1962; Cooper, 1966 and 1967). The greater TNC concentration in alfalfa roots relative to trefoil roots (Smith 1962; Barta, 1978) may have been the factor contributing to the differential responses in nodule activity with shading treatment.
Table 12. Alfalfa and birdsfoot trefoil nodule activity and dry weight after 80% shading for 7 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acetylene reduction</th>
<th>Nodule dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol·h⁻¹·pot⁻¹</td>
<td>nmol·h⁻¹·mg⁻¹</td>
</tr>
<tr>
<td>alfalfa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>42.7</td>
<td>87.1</td>
</tr>
<tr>
<td>shading</td>
<td>5.6</td>
<td>22.6</td>
</tr>
<tr>
<td>birdsfoot</td>
<td>trefoil</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>22.9</td>
<td>34.5</td>
</tr>
<tr>
<td>shading</td>
<td>1.9</td>
<td>5.7</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>13.6</td>
<td>21.8</td>
</tr>
</tbody>
</table>
Nodule dry weight of trefoil was reduced significantly by shading, while alfalfa nodule dry weight was reduced relatively less (Table 12). The different responses of nodule mass to shading between species seem to suggest different mechanisms of nodule growth and senescence pattern. The greater reduction of nodule dry weight of trefoil under shading suggests that trefoil nodules were sloughed off due to the transient nature of the determinate meristem (Vance et al., 1982). Senescence of nodules might have caused the reduced nodule activity in alfalfa nodules, but due to the nature of indeterminate meristem, alfalfa nodules may survive the shading treatment (Vance et al., 1980).

II. \( ^{14}\text{C}-\text{photosynthate partitioning} \)

The mean total radioactivity recovered per pot was 12.7\( \times 10^6 \)dpm for the control plants and 5.7\( \times 10^6 \)dpm for the shaded plants. There was no difference between the radioactivity recovered in alfalfa and trefoil, but there was a significant difference (p<.05) between the control and shaded plants suggesting that photosynthesis was possibly inhibited due to shading. Partitioning of \( ^{14}\text{C} \) photosynthate among shoots, roots, and nodules in the shading experiment (Table 13) was not the same as that
Table 13. Percentage $^{14}$C recovered in alfalfa and birdsfoot trefoil shoots, roots, and nodules exposed to 80% shade for 7 days. Plants were harvested 2 h after a 30 min pulse-labeling.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot</th>
<th>Root</th>
<th>Nodule</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>% total $^{14}$C recovered</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alfalfa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>96.48</td>
<td>2.75</td>
<td>0.78</td>
</tr>
<tr>
<td>shading</td>
<td>96.83</td>
<td>3.65</td>
<td>0.12</td>
</tr>
<tr>
<td>birdsfoot trefoil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>94.08</td>
<td>3.68</td>
<td>2.24</td>
</tr>
<tr>
<td>shading</td>
<td>96.78</td>
<td>2.59</td>
<td>0.63</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>1.71</td>
<td>N.S.</td>
<td>0.56</td>
</tr>
</tbody>
</table>
observed in the time course (Fig. 3) or dark experiments (Table 9). Although the chase time for the shading experiment was 2 hours, the percentage $^{14}$C recovered in the shoots was very high, compared with the $^{14}$C recovered in the shoots of the dark experiment or the time course experiment. Due to the better plant growth in early summer, plant total dry weights of shading experiment were two to six times higher than dark and time course experiment, respectively (Appendix A, B, and C). Hence, the pattern of photosynthate partitioning may be altered due to the difference in growth stage (Wolf, 1967) and also by a longer translocation path.

Nodules of both species were significantly affected by shading. The dark depletion experiment (Table 9 and 10) shows that nodules are strong sinks under normal conditions, but nodules fail to compete effectively for photosynthate when there is a shortage in photosynthate supply. The reduction of percentage $^{14}$C accumulation in trefoil nodules by shading also supports this hypothesis.

Both alfalfa and trefoil nodule RSA were reduced significantly by shading and were reduced by a greater magnitude than root RSA (Table 14). The greater than 3 fold reduction in nodule RSA (Table 14) is similar to that reported in pea nodules in response to shading.
Table 14. Relative specific radioactivity (RSA) of alfalfa and birdsfoot trefoil exposed to 80% shade for 7 days. Two month old plants were pulse-labeled for 30 min and harvested 2 h after labeling. RSA equals the specific radioactivity (SR) of an organ divided by the mean SR of the whole plant.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot</th>
<th>Root</th>
<th>Nodule</th>
<th>RSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>alfalfa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>1.47</td>
<td>0.09</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>shading</td>
<td>1.41</td>
<td>0.10</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>birdsfoot trefoil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>1.37</td>
<td>0.18</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>shading</td>
<td>1.33</td>
<td>0.11</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>LSD(05)</td>
<td>0.09</td>
<td>0.04</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>
(Sheikholeslam et al., 1980). Contrary to the time course and dark depletion experiments, RSA of trefoil control nodules was higher than the nodule RSA of the alfalfa control (Table 14). A possible reason for the observed difference in nodule RSA may be due to the altered partitioning pattern caused by the difference in the growth stage (Wolf, 1967) and a longer translocation path.

Table 15 shows the percentage $^{14}$C distribution in seven nodule metabolite fractions. An interesting comparison between alfalfa nodules and trefoil nodules was that 80% shading for 7 days had little effect on the metabolite partitioning pattern in trefoil nodules, but was significant in most alfalfa fractions.

The change in the $^{14}$C distribution pattern in alfalfa nodule fractions may have been due to the higher demand of alfalfa nodules for carbohydrates to support nodule activity (Table 12). There was a significant reduction in $^{14}$C activity in the sugar fraction and a significant increase in the organic acid fraction of alfalfa nodules (Table 15). This change in alfalfa nodules indicates a rapid turnover of incoming sugars, and suggests that shaded alfalfa nodules maintain a higher nodule activity, compared to the shaded trefoil nodules (Table 12), depending more on the current photosynthate.
Table 15. Percentage $^{14}$C recovered in alfalfa and birdsfoot trefoil nodule fractions exposed to 80% shade for 7 days. Plants were pulse-labeled for 30 min and harvested 2 h after labeling.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sugar</th>
<th>Organic acid</th>
<th>Amino acid</th>
<th>Starch</th>
<th>Protein</th>
<th>Chloro-Residue form</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>alfalfa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>45.44</td>
<td>10.60</td>
<td>35.83</td>
<td>4.19</td>
<td>1.45</td>
<td>2.05</td>
</tr>
<tr>
<td>shading</td>
<td>30.69</td>
<td>20.06</td>
<td>33.87</td>
<td>6.62</td>
<td>2.93</td>
<td>5.02</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>10.71</td>
<td>5.06</td>
<td>N.S.</td>
<td>1.07</td>
<td>0.48</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>birdsfoot trefoil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>41.78</td>
<td>16.27</td>
<td>28.36</td>
<td>6.62</td>
<td>2.17</td>
<td>1.66</td>
</tr>
<tr>
<td>shading</td>
<td>41.23</td>
<td>18.12</td>
<td>29.08</td>
<td>5.81</td>
<td>2.36</td>
<td>2.83</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>0.78</td>
</tr>
</tbody>
</table>

$^{14}$C recovered in the nodule
Effect of nodule size

The proportion of nodules found in three nodule size classes is presented in Table 16. Alfalfa had similar nodule mass in all three sizes. Small trefoil nodules (having diameter less than 1 mm) had significantly more nodule dry weight than medium and large nodules (Table 16).

Nodule size had a significant effect on nodule activity (Table 16). Small nodules of alfalfa had the highest specific nodule activity (SNA), which is similar to the value reported by Paau and Cowles (1981) for small detached alfalfa nodules. Small elongate nodules have the least senesced tissues and the percentage senesced tissues increase as the nodule size increase (Paau and Cowles, 1981). Thus, the effect of size on the elongate nodule of alfalfa can be attributed to the different proportions of senesced nodule tissues.

In contrast to alfalfa nodules, spherical trefoil nodules of medium size had the highest SNA (Table 16). Small trefoil nodules are white and contain numerous actively dividing cells, prolific infection thread development, and may be not fully functional in nitrogen fixation (Vance et al., 1982). Large trefoil nodules have increasing numbers of inactive, senesced cells (Bond,
Table 16. Alfalfa and birdsfoot trefoil nodule dry weight and specific nodule activity (SNA) in three nodule size classes. Nodule dry weight data were obtained from the shading experiment. SNA was measured by the acetylene reduction assay using detached nodules. FW.: fresh weight.

<table>
<thead>
<tr>
<th>Nodule size</th>
<th>Nodule dry weight</th>
<th>SNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>( \mu M C_2H_2 \cdot h^{-1} \cdot g^{-1} ) FW.</td>
</tr>
<tr>
<td>alfalfa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>small</td>
<td>128.4</td>
<td>11.6</td>
</tr>
<tr>
<td>medium</td>
<td>187.7</td>
<td>4.1</td>
</tr>
<tr>
<td>large</td>
<td>228.1</td>
<td>2.5</td>
</tr>
<tr>
<td>LSD(05)</td>
<td>N.S.</td>
<td>5.6</td>
</tr>
<tr>
<td>birdsfoot trefoil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>small</td>
<td>382.2</td>
<td>2.6</td>
</tr>
<tr>
<td>medium</td>
<td>237.8</td>
<td>4.3</td>
</tr>
<tr>
<td>large</td>
<td>11.8</td>
<td>1.7</td>
</tr>
<tr>
<td>LSD(05)</td>
<td>139.8</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*nodule size for alfalfa: small, shorter than 3 mm and unbranched; medium, longer than 3 mm and unbranched, or shorter than 3 mm and branched; large, longer than 3 mm and branched. Nodule size for trefoil: small, diameter less than or equal to 1 mm; medium, diameter greater than 1 mm and less than 2 mm; large, diameter greater than 2 mm.
1941). Hence, spherical nodules of medium size tend to have the highest SNA such as reported in soybeans (Mague and Buriss, 1972) and *Vicia faba* (Lawrie and Wheeler, 1975). The greater mass and the lower SNA in small nodules are the probable reason for the overall poor performance of SNA observed in trefoil nodules (Table 2, 7, and 12).

Control alfalfa nodules contained about six fold more radioactivity than shaded nodules (Table 17), but radioactivity was not significantly affected by nodule sizes. The effect of shading on alfalfa nodules was similar to earlier data showing that alfalfa nodules are strong sinks under normal light conditions (Table 14), but the significant decline in SR by shading suggests that nodules may be sinks of low priority. Thus, when there is a shortage of photosynthate, alfalfa nodules lose their competitiveness to other plant organs.

Nodule size had a significant effect on $^{14}$C photosynthate partitioning to trefoil nodules. Under control conditions, small trefoil nodules had 2 and 4 times more radioactivity than medium nodules and large nodules, respectively (Table 17). This indicates that small trefoil nodules are stronger sinks than medium and large nodules. The greater meristematic activity in small nodules may
Table 17. Alfalfa and birdsfoot trefoil nodule specific radioactivity (SR) in three nodule size classes after exposed to 80% shade for 7 days. Two month old plants were pulse-labeled for 30 min and harvested 2 h after labeling.

<table>
<thead>
<tr>
<th>Nodule* size</th>
<th>Specific radioactivity (SR)</th>
<th>dpm·mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>alfalfa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>small</td>
<td>143.8</td>
<td>20.2</td>
</tr>
<tr>
<td>medium</td>
<td>138.1</td>
<td>18.2</td>
</tr>
<tr>
<td>large</td>
<td>107.6</td>
<td>19.9</td>
</tr>
<tr>
<td>birdsfoot trefoil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>small</td>
<td>409.8</td>
<td>43.5</td>
</tr>
<tr>
<td>medium</td>
<td>182.1</td>
<td>20.6</td>
</tr>
<tr>
<td>large</td>
<td>91.5</td>
<td>3.3</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>61.6</td>
<td>12.0</td>
</tr>
</tbody>
</table>

*nodule size classification is the same as Table 16.
contribute to their greater sink strength, since their SNA activities are low. Shading had a severe effect on large trefoil nodules. Specific radioactivity (SR) of large nodules was reduced to less than 4% of the control, while medium and small nodules were reduced to about 10% of control (Table 17). The reduction in trefoil large nodule assimilate accumulation may provide more photosynthates for the use by small and medium nodules. The reduction of trefoil nodule dry weight by shading treatment (Table 12), the significantly lower SNA of large trefoil nodules (Table 16), and the selective reduction in SR of trefoil large nodules (Table 17) suggest that large nodules might have been sloughed off roots.

Strategies for adapting to photosynthate deficiency appear to be different for alfalfa and trefoil nodules. Indeterminate meristems and the relative low reduction in nodule dry weight by shading (Table 12) suggest that alfalfa nodules are more able to survive stress. The data reported here support the suggestion by Vance et al. (1982) that trefoil nodules with determinant growth may be less efficient in nodule growth and function than alfalfa nodules with indeterminate growth.
Nodule respiration studies

Specific nodule activity of alfalfa nodules with sand removal by shaking off the root was nearly double when compared to washing sand off the root (Table 18). Washing had no effect on nodule activity of trefoil (Table 18). Cralle and Heichel (1982) also observed a reduction of alfalfa SNA in response to washing and attributed the reduction of alfalfa SNA to the low temperature caused by the washing water. However, water used in this experiment to wash roots was at room temperature (21°C), thus temperature was probably not the reason for the reduced SNA rates, also a similar reduction should be observed in trefoil nodules, if the reduction of nodule activity was caused by water temperature. The washing effect on alfalfa nodule activity could have been due to the layer of water adsorbed onto the nodule surface retarding O₂ permeation.

In order to test whether oxygen availability affected nodule function, nodule respiration rates were measured (Table 19). Whole nodule respiration rates of alfalfa nodules were about twice that of trefoil nodules. The greater respiration rate of alfalfa nodules is probably necessary to support the greater SNA observed in alfalfa
Table 18. The effect of shaking or washing sand off root systems on alfalfa and birdsfoot trefoil nodule activities. S.E.: standard error. DW.: dry weight.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acetylene reduction rate</th>
<th>nM·h⁻¹·mg⁻¹ nodule DW.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM·h⁻¹·pot⁻¹</td>
<td></td>
</tr>
<tr>
<td>alfalfa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>shaking</td>
<td>21.00</td>
<td>107.08</td>
</tr>
<tr>
<td>washing</td>
<td>14.82</td>
<td>58.18</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>N.S.</td>
<td>13.67</td>
</tr>
<tr>
<td>S.E.</td>
<td>4.81</td>
<td>3.48</td>
</tr>
<tr>
<td>birdsfoot trefoil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>shaking</td>
<td>17.38</td>
<td>26.25</td>
</tr>
<tr>
<td>washing</td>
<td>14.73</td>
<td>23.40</td>
</tr>
<tr>
<td>S.E.</td>
<td>2.52</td>
<td>2.35</td>
</tr>
</tbody>
</table>
Table 19. Oxygen uptake rates of detached alfalfa and birdsfoot trefoil nodules as measured by an oxygen electrode. Values are the mean of 3 replicates ± standard error.

<table>
<thead>
<tr>
<th>Species</th>
<th>Whole nodule</th>
<th>Sliced nodule</th>
</tr>
</thead>
<tbody>
<tr>
<td>alfalfa</td>
<td>204±21</td>
<td>291±16</td>
</tr>
<tr>
<td>birdsfoot trefoil</td>
<td>122±9</td>
<td>241±19</td>
</tr>
</tbody>
</table>
nODULES (Table 2 and 7). However, similar to the effect of increasing O$_2$ partial pressure around soybean nodules reported by Pankhurst and Sprent (1976), when nodules were sliced, O$_2$ uptake rates of birdsfoot trefoil nodules were doubled to the similar level of alfalfa nodules, while O$_2$ uptake rates of alfalfa nodules were only increased slightly by slicing (Table 19). Paau and Cowles (1978) did not find any difference in nodule activity of whole or sliced alfalfa nodules. However, sliced lupin nodules were reported to have 5 fold increase in nodule activity (Sutton and Jepsen, 1975) in relative to whole nodules. Thus the data shown here strongly suggests that O$_2$ flux in trefoil nodules may be limiting their activity.

Moisture on the nodule surface is important to maintain lenticel openings (Pankhurst and Sprent, 1976). This may explain why nodule activity of trefoil was not affected by washing. Trefoil nodule tissues also have a higher number of bacteroids per unit nodule volume (Vance et al., 1982), compared with alfalfa nodules. This may result in an intranodular oxygen deficiency preventing higher expression of bacteroidal nitrogenase activity as suggested by Sen and Weaver (1984). Furthermore, there is a band of flavolan-containing cells in the outer trefoil nodule cortex only interrupted by lenticel tissues (Pankhurst,
Craig, and Jones, 1979). This band may restrict O₂ exchange. Slicing trefoil nodules relieves this O₂ diffusion barrier and may contribute to the doubling of O₂ uptake rates.

Minchin, Witty, Sheehy, and Muller (1983) and Sheehy, Minchin, and Witty (1983) suggested that alfalfa nodules control O₂ flux by varying resistance through cortical tissues, while trefoil nodules control O₂ diffusion through the lenticels, which may be controlled by the pressure of nodule vascular system (Ralston and Imsande, 1982). The present data suggest that trefoil nodules, which depend on lenticels for aeration, may be under O₂ limiting conditions.

**CN-resistant respiration of bacteroids**

Oxygen uptake due to cyanide (CN)-resistant respiration was present in both species and comprised 59 and 94% of control respiration in alfalfa and trefoil respectively (Table 20). Oxygen uptake by the alternate electron pathway, SHAM (salicylhydroxamic acid)-sensitive, was about 45% for alfalfa bacteroids and 21% for trefoil bacteroids (Table 21). Others have also reported on CN-insensitive respiration in legumes. Emerich, Ruiz-Argueso, Russell, and Evans (1980) found 37% inhibition of soybean bacteroid
Table 20. The effect of KCN on oxygen uptake of alfalfa and birdsfoot trefoil bacteroids. Values are mean ± standard error. Numbers in the parenthesis are expressed as percentage of control.

<table>
<thead>
<tr>
<th>Source of bacteroid</th>
<th>O₂ uptake rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>+0.2mM KCN</td>
</tr>
<tr>
<td></td>
<td>nM O₂·min⁻¹·g⁻¹ nodule fresh weight</td>
</tr>
<tr>
<td>alfalfa</td>
<td>62.91± 4.27 (100%)</td>
</tr>
<tr>
<td>birdsfoot trefoil</td>
<td>84.16±10.04 (100%)</td>
</tr>
</tbody>
</table>
Table 21. The effect of salicylhydroxamic acid (SHAM) and KCN on O$_2$ uptake of alfalfa and trefoil bacteroids.
Values are mean ± standard error. Numbers in the parenthesis are expressed as percent of control.

<table>
<thead>
<tr>
<th>Source of bacteroid</th>
<th>Control</th>
<th>+1.0 mM SHAM</th>
<th>+2.0 mM KCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>alfalfa</td>
<td>32.71±1.00 (100%)</td>
<td>18.02±1.15 (55%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>birdsfoot</td>
<td>47.52±1.88 (100%)</td>
<td>25.74±2.83 (79%)</td>
<td>25.74 (79%)</td>
</tr>
<tr>
<td>trefoil</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

nM O$_2$·min$^{-1}$·g$^{-1}$ nodule fresh weight
respiration, while Day, Arron, and Laties (1980) reported respiration resistant to both CN and SHAM in plant roots.

The CN-sensitive pathway is a TCA cycle-implemented, cytochrome oxidase-mediated energy producing pathway. Because nitrogen fixation has high energy requirements, activity of this pathway would appear to be critical for efficient nitrogen fixation. CN-resistant respiration is uncoupled respiration and does not produce ATP. Since the H\textsubscript{2} oxidation reaction by hydrogenase in bacteroids is also inhibited by KCN, the CN-resistant respiration was not due to electron flow through hydrogenase (Emerich et al., 1980). It is possible that a protective mechanism was triggered during aerobic extraction of bacteroids or this uncoupled respiration might be present in vivo to protect nitrogenase under high O\textsubscript{2} concentration (Appleby, 1984).

These data suggest that alfalfa nodules may be more efficient in energy production since a greater portion of their O\textsubscript{2} uptake appears to be coupled to oxidative phosphorylation. The reduced rates of symbiotic nitrogen fixation observed in trefoil nodules could be in part due to their greater activity of CN-insensitive respiration resulting in less efficient use of carbon for energy production.
GENERAL DISCUSSION

The data relating to photosynthetic capacity collected in this study (Table 1) as well as previous reports in the literature suggest that alfalfa is capable of fixing a significantly greater amount of carbon per plant or unit leaf area than is birdsfoot trefoil. This difference in photosynthate supply could be a major cause of the low nitrogen fixation rates observed in trefoil nodules.

Alfalfa shoots transported more starch from their leaves during a diurnal cycle than did trefoil (Fig. 2c). Because these species have been reported to maintain relatively high nitrogen fixation rates during the dark, alfalfa may be able to supply more carbohydrates to nodules during this time.

Alfalfa shoots not only fixed more carbon but also translocated a greater proportion of their photosynthate to their roots than did trefoil shoots (Fig. 3) resulting in greater TNC in alfalfa roots. The greater TNC content in the roots could be used by nodules for nitrogen
fixation, especially during periods of low photosynthate supply.

Sink strength as estimated by specific radioactivity and RSA data suggests that alfalfa nodules are stronger sinks for current photosynthate than trefoil nodules (Table 4 and Fig. 4). Although birdsfoot trefoil nodules did not have high sink strength, relative to alfalfa, they had a higher 'mobilizing ability' than alfalfa nodules since they accumulated more nodule mass (Table 2). However, with regards to nitrogen fixation, a greater sink strength appears to be more beneficial than a greater 'mobilizing ability'.

Sink strength estimated by specific radioactivity and RSA data suggests that shoots of both species are the most dominant sinks (Fig. 4, Table 4, 10, and 14). Compared to roots, nodules are stronger sinks, since nodule RSA (Table 10 and 14) is higher than root RSA under control conditions and nodule RSA is about the same as roots under dark or shading conditions. Thus, nodules may have strong sink strength under normal conditions, but nodules are probably sinks of low priority when photosynthate supply is low.

The flux of photosynthate to alfalfa nodules is higher, since alfalfa nodules have higher RSA than trefoil nodules. Therefore, alfalfa nodules have a greater amount of photosynthate available for nitrogen fixation.
Additionally, alfalfa nodules used a lower proportion of fixed $^{14}\text{C}$ for growth, hence there was relatively more photosynthate for nitrogen fixation. The observation that more $^{14}\text{C}$ was partitioned into the nodule residue fraction (Fig. 9 and Table 6) and small nodules (Table 17) of trefoil suggests that birdsfoot trefoil depends upon continuous initiation of new nodules to maintain nitrogen fixation (Fig. 5). The large nodule mass of birdsfoot trefoil has been suggested to be a possible survival strategy to compensate for the loss of nodules under stress (Vance et al., 1982).

The question of whether carbohydrate supply or oxygen availability most limits nitrogen fixation has not yet been resolved. Relatively high carbohydrate concentration in senesced nodules does not favor carbohydrate limiting nitrogen fixation (Martinez-Rodas, 1977; Streeter, 1981). Minchin (1984) has suggested that $O_2$ availability is more important than carbohydrate supply in nitrogen fixation. The importance of oxygen availability is also supported by the respiratory data of this experiment (Table 19) when the low nitrogen fixation of birdsfoot trefoil nodules appeared to be related to the deficiency of oxygen.

Respiratory studies suggest that bacteroids of birdsfoot trefoil nodules may be less efficient in terms of energy conversion due to lower CN-sensitive respiration.
(Table 20 and 21). Although, the evidence is not conclusive that coupling of the electron transfer chain to phosphorylation in bacteroids of birdsfoot trefoil was low, the data provided in Table 20 and 21 suggest that bacteroids of alfalfa and birdsfoot trefoil differ in their efficiencies of energy production. The respiration of bacteroids and nodules should be further explored, since respiration is the key mechanism providing energy for nitrogen fixation.

The results of this study strongly suggest that higher nitrogen fixation capacity in alfalfa relative to that in birdsfoot trefoil is not due to a greater nodule mass, but due to higher photosynthate supply to the nodule and more efficient energy production from metabolism of these photosynthates. The differential distribution of photosynthate to different sizes of nodules suggests that nodule development and morphology may play an important role on nitrogen fixation.

According to Zhiznevskaya et al. (1979), legumes with spherical nodules, such as birdsfoot trefoil, are phylogenetically older than legumes with cylindrical nodules, such as alfalfa. There is overwhelming evidence that phylogenetical younger nodules are more advanced and have higher nitrogen fixation efficiency based on unit nodule dry weight (Dart and Day, 1971; Zhiznevskaya et
al., 1979). Two species examined in this research appear to exemplify this phylogenetical relationship. If this hypothesis holds true, then the improvement of nitrogen fixation for legumes of different nodule shapes should adopt different strategies. For the spherical nodule species, an increased supply of photosynthates and oxygen to the nodules may be beneficial. In the case of cylindrical nodule species, such as alfalfa, increasing nodule numbers may be a better approach.
Appendix A. Component dry weight of plants used in the time course experiment.

<table>
<thead>
<tr>
<th>Species</th>
<th>Shoot</th>
<th>Root</th>
<th>Nodule</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>alfalfa</td>
<td>2229.3</td>
<td>2636.0</td>
<td>94.1</td>
<td>5029.5</td>
</tr>
<tr>
<td>trefoil</td>
<td>1922.0</td>
<td>1145.3</td>
<td>155.0</td>
<td>3222.3</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>303.5</td>
<td>321.8</td>
<td>13.4</td>
<td>543.8</td>
</tr>
</tbody>
</table>
Appendix B. Alfalfa and birdsfoot trefoil component dry weight after 40 hours of dark treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot</th>
<th>Root</th>
<th>Nodule</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>alfalfa</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>7063.3</td>
<td>6231.7</td>
<td>302.7</td>
<td>17907.0</td>
</tr>
<tr>
<td>dark</td>
<td>6166.7</td>
<td>5126.7</td>
<td>219.3</td>
<td>16006.3</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>769.0</td>
<td>1124.9</td>
<td>42.9</td>
<td>1697.8</td>
</tr>
<tr>
<td><strong>birdsfoot trefoil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>7565.0</td>
<td>3950.0</td>
<td>657.9</td>
<td>16137.0</td>
</tr>
<tr>
<td>dark</td>
<td>6700.0</td>
<td>3880.0</td>
<td>530.7</td>
<td>15302.0</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>N.S.</td>
<td>N.S.</td>
<td>113.6</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
Appendix C. Alfalfa and birdsfoot trefoil component dry weight after exposure to 30% shade for 7 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot</th>
<th>Root</th>
<th>Nodule</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>alfalfa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>22186.7</td>
<td>10876.1</td>
<td>770.8</td>
<td>33834.1</td>
</tr>
<tr>
<td>shading</td>
<td>14696.7</td>
<td>6443.3</td>
<td>317.5</td>
<td>21457.5</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>4649.8</td>
<td>986.6</td>
<td>196.1</td>
<td>4945.5</td>
</tr>
<tr>
<td>birdsfoot trefoil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>13976.7</td>
<td>3790.0</td>
<td>906.9</td>
<td>18673.5</td>
</tr>
<tr>
<td>shading</td>
<td>5680.0</td>
<td>1763.3</td>
<td>356.6</td>
<td>7799.9</td>
</tr>
<tr>
<td>LSD(.05)</td>
<td>6322.6</td>
<td>336.7</td>
<td>161.7</td>
<td>6561.2</td>
</tr>
</tbody>
</table>

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