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METABOLIC CHANGES IN SENESCING SOYBEAN LEAVES

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Metabolic changes in senescing soybean leaves

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INTRODUCTION

The increase in the demand for food caused by an increasing population and a decreasing arable land area requires a higher yield per land area. This, coupled with decreasing reserves of fossil fuels, suggests that crop production should be viewed in terms of energy inputs. The full exploitation of the most abundant and least costly energy supply available--the sun--has yet to be achieved. Improving photosynthesis is one means of utilizing solar energy better.

The improvement of the photosynthetic efficiency of a crop can be accomplished by optimizing the environmental conditions or maximizing the internal biochemical efficiency of the plant. An early decline in leaf photosynthesis may be a biological inefficiency that can be improved. This study seeks to identify metabolic parameters that are associated with the decline in leaf photosynthesis.

From a functional, physiological point of view, I believe that leaf senescence commences when leaf photosynthesis begins to decline. There is convincing evidence that delaying this decline in photosynthesis, or the beginning of senescence, results in higher yields. Hardy et al. (1978), citing several examples where a positive correlation exists between the longevity of the flag leaf and yield in wheat (Triticum aestivum L.), noted that senescence is one of the four types of rate limitations related to photosynthesis "that offer

the potential for 20-50% increase in productivity." They theorized that an increase in yield of about 3% per day would be expected for each day senescence is delayed in wheat. Increased yield in soybeans [Glycine max (L.) Merr.] has been demonstrated by maintaining high photosynthesis through carbon dioxide enrichment (Hardman and Brun, 1971; Hardy et al., 1978). Naturally occurring delayed senescence that may lead to an eventual increase in soybean yield has been recently reported (Abu-Shakra et al., 1978).

The study of senescence and the factors involved with its initiation requires the integration of many facets of plant physiology. Descriptive investigations have pointed out that the loss of protein and chlorophyll are among the earliest symptoms associated with foliar senescence (Martin and Thimann, 1972; Wittenbach, 1978). Yet, as Wittenbach (1980) stated, "the factor(s) responsible for the initiation of plant senescence is unknown." And, according to Thomas and Stoddart (1980), "It is a chastening thought that it is not yet possible to explain in enzymic terms the cause of a single metabolic or physiological change that occurs in senescent leaves." Because senescence has been studied in widely varying types, conditions and ages of plant material, it is easy to understand why little is understood about its mechanism.

Although Hall et al. (1978) believe that information from intact plants undergoing natural senescence due to reproduc-

tive maturity may be the most relevant to crop productivity, I believe that an understanding of the senescence processes within leaves developing throughout plant ontogeny is equally relevant to crop productivity. The maintenance of high photosynthesis in older leaves, as well as in younger leaves, is essential for maximizing yield. By understanding the mechanisms involved in senescence, the retention of maximum photosynthesis can be eventually accomplished.

The objective of this study is to characterize leaf metabolic patterns during senescence.

LITERATURE REVIEW

Senescence is a ubiquitous process, occurring not only in the plant as a whole, but also within the constitutive elements of the plant--organelles, cells, tissues and organs. Yet senescence remains a scientifically vague and undefined process because of its complex nature (Nooden and Leopold, 1980; Thomas and Stoddart, 1980). Moreover, senescence often is confused with a closely related process--ageing (Wareing and Seth, 1967). Ageing should be restricted to mean chronological development (Carr and Pate, 1967; Beevers, 1976; Wittenbach, 1979), whereas senescence refers specifically to the deteriorative events preceding death (Carr and Pate, 1967; Beevers, 1976; Nooden and Leopold, 1980; Wittenbach, 1979). Leaf senescence is a distinct type of senescence involving primarily leaf mesophyll cells. Thomas and Stoddart (1980) have defined leaf senescence as "the series of events concerned with cellular disassembly in the leaf and the mobilization of materials released during this process." They and others (Brun, 1980; Wittenbach, 1980) have emphasized that leaf senescence is a closely integrated syndrome consisting of an ordered sequence of genetically controlled events. But the sequence of events and the genetic control remain an enigma.

The initiation of the senescence program is delayed until an appropriate stage in leaf development. When leaves do

begin to senesce on a plant, they may do so under and because of different circumstances. These circumstances form a basis for the classification of leaf senescence (Simon, 1967; Woolhouse, 1967; Beevers, 1976; Thomas and Stoddart, 1980). Three general causal mechanisms and categories of leaf senescence can be recognized: environmental, endogenous, and correlative.

Environmental leaf senescence can be brought about through influences exerted by light, temperature, water, nutrient levels, and pathogens (Woolhouse, 1967; Thomas and Stoddart, 1980). Environmentally induced senescence is a real and important phenomenon, occurring frequently in field-grown plants. This is a difficult process to characterize accurately because of the many internal and external influencing factors.

Endogenous (Woolhouse, 1967) or genetic (Thomas and Stoddart, 1980) leaf senescence arises from an internally initiated, preprogrammed genetic message coding for the self-destruction of the leaf. Inherent in the definition of this type of leaf senescence is that the leaf behaves independently of the rest of the plant. Thus, it is implied that the leaf regulates its own senescence. There is convincing evidence that the nuclear, rather than the chloroplastic, genome regulates cellular senescence. However, the initiating factor is unknown (Thomas and Stoddart, 1980). The

existence of purely endogenous senescence has been questioned by Wareing and Seth (1967) who believe that "the rate of senescence of plant organs is often under the control of the whole plant and is not simply determined by intrinsic characteristics of the cells of that organ."

Correlative senescence is based upon the assumption that there is communication between remote organs of a plant, resulting in competition among organs for space, light, or growth regulators (Thomas and Stoddart, 1980). This competition causes and may regulate the rate of leaf senescence. Correlative senescence can occur during both the vegetative and reproductive phases of plant development, but the metabolic characteristics of leaf senescence may differ between the two developmental phases. As Simon (1967) pointed out,

> An upper leaf which senesces and dies after the initiation of the reproductive phase may outwardly appear to have gone through the same sequence of changes of a leaf which senesces and dies while the plant is in a purely vegetative phase. However, it must be recognized that without adequate evidence, it would be foolish to assert that senescence had been the same in each case.

Evidence, although circumstantial, that the nature of leaf senescence may differ between developmental stages is that, in vegetative plants, leaves usually senesce acropetally and sequentially (Simon, 1967), whereas leaves more or less synchronously senesce during the later stages of the reproductive period. This special type of correlative senescence occurring during late reproduction is known as monocarpic senescence.

Monocarpic senescence is characterized by a single flowering phase followed by leaf senescence and subsequent plant death. Thus, monocarpic senescence is a whole-plant phenomenon that is most obviously manifested in leaves initially. In crop species harvested for seed, this type of senescence is desirable because of the ease of harvesting dry, leafless plants (Nooden and Lindoo, 1978). The relationship between leaf senescence and pollination, fruiting and flowering has been the focus of many investigations (cf. Nooden and Leopold, 1980). The removal of the reproductive organs is known to delay senescence in certain species (Leopold et al., 1959; Wareing and Seth, 1967; Woolhouse, 1967; Lindoo and Nooden, 1977), whereas in other species, notably maize (Zea mays L.) (Allison and Weinmann, 1970), barley (Hordeum vulgare L.) (Mandahar and Carg, 1975), and pepper (Capsicum annum L.) (Hall and Brady, 1977), leaf senescence is accelerated by removal of young reproductive organs. Clearly, then, there are different monocarpic mechanisms operating in different species.

Nooden and his coworkers (Lindoo and Nooden, 1977, 1978; Derman et al., 1978; Nooden and Lindoo, 1978; Nooden et al., 1978) have studied extensively monocarpic senescence in soybeans [Glycine max (L.) Merr.]. They have concluded from their studies that developing soybean seeds generate a "death" signal, which is the cause of monocarpic senescence.

This signal is emitted only during the final stage of seed development and moves in a basipetal direction. What effect the signal has on the initiation and regulation of leaf senescence is unknown.

Metabolic Nature of Senescence

Photosynthesis

Photosynthesis is a physiological process involving physical, photochemical and biochemical parameters and, as such, is subject to complex regulation and metabolic interactions. According to Beevers (1976), there have been very few studies aimed at discovering the sequence in which the partial reactions of photosynthesis become restricted. It is known that, in general, as a leaf grows and chloroplasts develop, there is a rapid increase in the rate of carbon fixation to a maximum at, or just before, the time of full leaf expansion (Catsky et al., 1976; Woodward, 1976; Woodward and Rawson, 1976; Woolhouse, 1978; Patterson and Moss, 1979; Thomas and Stoddart, 1980), or sometime after full leaf expansion is reached (Dornhoff and Shibles, 1974; Bethlenfalvay and Phillips, 1977; Wittenbach et al., 1980).

The decline in photosynthesis after reaching its maximum can vary in rate and time. A steady rate of decline is observed most often (Hopkinson, 1964; Woolhouse, 1974; Catsky et al., 1976; Patterson and Moss, 1979; Wittenbach et al.,

1980). Woodward and Rawson (1976) reported that photosynthesis in the fourth trifoliolate soybean leaf began to decline steadily but experienced two transient peaks corresponding to the time of flowering and pod filling. Transient peaks during the period of declining photosynthesis in the primary and the first three trifoliolate leaves of Phaseolus vulgaris L. also have been observed (Fraser and Bidwell, 1974).

Leaves at different positions on the plant attain different maximum photosynthetic rates and have different rates of decline in photosynthesis (Kumura and Naniwa, 1965; Woodward, 1976; Patterson and Moss, 1979). In general, the leaves in the upper canopy reach a higher maximum level of photosynthesis, but no clear-cut trend is evident for the decline in photosynthesis. In wheat (Triticum aestivum L.), photosynthesis in the upper leaves declines at a faster rate than in the lower leaves (Patterson and Moss, 1979), whereas in the upper leaves of determinant soybean plants (Woodward, 1976), field bean and pea (Pisum sativum L.) (Bethlenfalvay and Phillips, 1977) photosynthesis declines more slowly than in the lower leaves.

Photosynthetic products

Carr and Pate (1967) generalized that one fairly stable feature of the photosynthetic apparatus is that there is no significant change in the percentage distribution of $^{14}CO_2$-fed radioactivity among photosynthetically derived amino acids

throughout the photosynthetic life of a leaf. But the immediate photosynthetic products have been observed to change. In tobacco (Nicotiana tabacum L.), a C_3 species, there is evidence of C_4 photosynthesis occurring in very young leaves (Kisaki et al., 1973). In maize, a C_4 species, a decline in the amount and a change in the predominate type of C_4 acids produced occurs as leaves age (Williams and Kennedy, 1978).

Photorespiration

The decrease in photosynthesis over time is believed by Woolhouse (1978) to be partly accounted for by a progressively increasing rate of photorespiration. Although an increase in photorespiration is observed in ageing maize (Williams and Kennedy, 1978) and field bean (Catsky et al., 1976) leaves, photorespiration is greatest in very young tobacco leaves (Kisaki et al., 1973). More ambiguity in the ontogenetic trend of photorespiration is demonstrated by a relatively constant rate of photorespiration, even though photosynthate rate declined in the flag leaves of wheat (Thomas et al., 1978). The discrepancies in photorespiratory behavior may be due to the peculiarities of the plant species or to the method employed to estimate photorespiration (Chollet, 1978).

Respiration

Changes in mitochondrial or dark respiration may partly account for changes in net carbon assimilation. Yet, no

definite, clear-cut trend in respiration rate has been demonstrated among plant species. Respiration has been reported to decline from 4.1 to 1.8 mg $CO_2 \cdot hr^{-1} \cdot dm^{-2}$ in the second trifoliolate soybean leaf as it ages (Silvius et al., 1978). A similar pattern exists in the primary leaves of bean (Catsky et al., 1976) and in tobacco leaves (Kisaki et al., 1973). In pepper, Perilla frutescens L., and wheat leaves, respiration is steady over time and is not great enough to explain the effect of time on net photosynthetic rate (Kannangara and Woolhouse, 1968; Hall and Brady, 1977; Feller and Erismann, 1978). In other studies, the onset of senescence has been associated with a large increase (Tetley and Thimann, 1974) or large decrease (James, 1953) in respiratory rate. The role that respiration rate plays in affecting ontogenetic changes in net carbon assimilation is probably minor. Changes in respiration rate are more likely to arise as a consequence of reduced photosynthesis, leading to an altered metabolic state in the leaf.

Diffusive resistance

Stomatal and mesophyll resistance to CO_2 flux are physical barriers that can influence and regulate photosynthetic rate. A consensus among many researchers is that ontogenetic changes in photosynthesis are not caused by changes in diffusive resistance (Catsky et al., 1976; Woolhouse and Batt, 1976; Woodward and Rawson, 1976; Wittenbach et al., 1980)

though stomatal and mesophyll resistances do fluctuate over the season. In senescing barley leaves, stomatal resistance increases more rapidly, accounting for 24% of the total increase in resistance to CO_2 diffusion, than does mesophyll resistance (Friedrich and Huffaker, 1980). But in the same study, it was mesophyll resistance that increased at the same rate that photosynthesis decreased; however, the authors concluded that other events, rather than changes in resistances, were responsible for the decline in leaf photosynthesis. In detached, dark-induced, senescing oat (Avena sativa L.) leaf sections, stomatal aperature is considered to be the principal controlling agent of senescence (Thimann and Satler, 1979a, b). The difference between excised and intact leaf responses may account for the finding by Thimann and Satler.

Chlorophyll

The decline in chlorophyll content as a leaf ages is the most characteristic visible feature of leaf senescence. As such, the loss of chlorophyll has been the sole basis for measuring the progression of leaf senescence in many studies. Although the light-dependent pathways of chlorophyll biosynthesis have been elucidated (Salisbury and Ross, 1979), the enzymology of chlorophyll breakdown during senescence is quite obscure (Thomas and Stoddart, 1980). In fact, it is questioned whether chlorophyll degradation is an enzymatic

process at all (Holden, 1966). Chlorophyllase, the enzyme once believed to catalyze chlorophyll degradation, may actually function primarily in chlorophyll synthesis (Shimizu and Tamaki, 1963). Other findings suggest that the various "chlorophyllase" reactions are the responsibility of two enzymes, one phytolating and the other hydrolytic (Ellsworth et al., 1976), or that chlorophyllase is only active when in a reduced form and in a special conformation (Terpstra, 1977). Thus, *in vitro* assays may not truly represent *in vivo* activity.

The change in chlorophyll content over the life of a leaf is perhaps one of the most documented phenomena associated with senescence. Sestak (1977), in reviewing much of the literature concerning ontogenetic changes in chlorophyll content, stated that "generally during leaf ontogenesis chlorophyll accumulates to some maximal level, and afterwards the rate of degradative processes overtakes the rate of synthetic processes." Chlorophyll normally reaches a maximum level at, or near, the time of full leaf expansion, but the rate of decline after reaching the maximum level varies according to the leaf's position on the plant (Patterson and Moss, 1979).

The change in the ratio of chlorophyll a to chlorophyll b can indicate selective degradation of chlorophylls and of the photochemical reaction centers. Although Woolhouse

(1974) and Sestak (1977) have stated that the chlorophyll a:b ratio changes with time, neither in the primary leaves of bean (Sestak et al., 1977) nor in the upper (but not the lower) leaves of wheat (Patterson and Moss, 1979) does the chlorophyll a:b ratio change with time. The lower leaves of wheat do have a declining ratio with time. Using relatively sophisticated techniques, Melis and Brown (1980) have demonstrated that the ratio of photochemical centers, RCI and RCII, which are specialized chlorophyll a molecules connected in series to each other by intermediate electron transfer carriers, changes with time; developing pea chloroplasts have a higher RCII:RCI ratio than mature ones.

To be an accurate indicator of senescence, chlorophyll content should reflect sensitively the changes in the primary function of the leaf-photosynthesis. Certainly, if chlorophyll content and photosynthesis are not well linked then the change in leaf color poorly describes leaf senescence. The temporal relationship between photosynthesis and chlorophyll seems tenuous. In wheat, chlorophyll has been reported to begin to decline before (Feller and Erismann, 1978; Hall et al., 1978), after (Wittenbach, 1978) or coincident (Patterson and Moss, 1979) with the decline in photosynthesis. Similar discrepancies exist in soybean plants (Sesay and Shibles, 1980; Wittenbach et al., 1980).

Chlorophyll degradation is, because of ease

of measure, appealing to use as an estimate of senescence rate. But chlorophyll level can be separated from senescence. In comparing a nonyellowing mutant of meadow fescue (Festuca pratensis Huds.) to the normal genotype, Thomas and Stoddart (1975) learned that, with the exception of total chlorophyll loss, the mutant underwent all of the characteristics associated with normal senescence, including the loss of protein and ribonucleic acid (RNA). Other investigations using this mutant revealed that chlorophyll degradation is regulated by a gene or gene complex expressed only at senescence, but the activation of this gene and its expression in senescing leaves are not sequential in time (Thomas and Stoddart, 1977). Further evidence that senescence and chlorophyll content can be separated is that in depodded soybean plants, in which leaf chlorophyll content remained high, the leaves senesced at the same rate and at the same time as in the podded plants (Mondal et al., 1978). Thus, chlorophyll breakdown, which is used widely as an index of senescence, may not be an inevitable part of the ageing process.

Protein

Protein turnover, one means of metabolic regulation in plants, can be defined as the simultaneous synthesis and degradation of protein (Peterson et al., 1973), but because of the difficulty inherent in estimating simultaneous synthesis and degradation, Huffaker and Peterson (1974) redefined

turnover as the flux of amino acids through protein. The quantification of protein turnover is hampered by the channelling of amino acids into different metabolic and active pools. Other problems encountered in estimating and interpreting protein turnover are (1) changing rates and patterns of amino acid hydrolysis, (2) increasing rates of hydrolysis of newly synthesized product and (3) variable recycling of amino acids derived from protein degradation (Bidwell et al., 1964; Beevers, 1976).

In spite of the aforementioned considerations, protein turnover has been estimated in plant tissue. Experiments indicate that leaves retain the capacity to incorporate label derived from photosynthetically fixed $^{14}CO_2$, radioactive amino acids or ^{15}N-labelled compounds into protein throughout their life (Atkin and Srivastava, 1970; Stoddart, 1971; Hedley and Stoddart, 1972; Brady and Tung, 1975). The rate of protein turnover in leaves is not very great, estimated to be about 2% per hour (Hellebust and Bidwell, 1963; Simon, 1967). Nor is the rate of protein turnover constant over the life of a leaf. Hedley and Stoddart (1972) observed three maxima in the rate of incorporation of ^{14}C-labelled amino acids into protein during the development of Lolium leaves: during leaf expansion when chloroplasts are being assembled, in fully expanded leaves, and at the onset of senescence.

Ribulose bisphosphate carboxylase Protein is differentially turned over in plants. The amount of Fraction-I protein or ribulose 1,5-bisphosphate carboxylase (RuBPCase) (3-phospho-D-glycerate carboxy-lyase; EC4.1.1.39) decreases progressively from the time of full leaf expansion while the amount of Fraction-II protein (non-RuBPCase protein) decreases only in the later stages of senescence (Kannangara and Woolhouse, 1968). It has been shown subsequently that RuBPCase has a lower turnover rate than non-RuBPCase protein (Peterson et al., 1973; Nishimura and Akazawa, 1978). RuBPCase is an important constituent of plant protein, not only because it is the enzyme responsible for fixing CO_2, but also because it constitutes the major component of leaf soluble protein: 80% in barley (Friedrich and Huffaker, 1980), 50% in soybeans (Wittenbach et al., 1980) and 40% in wheat (Wittenbach, 1979). RuBPCase is synthesized en masse during leaf expansion and persists until the onset of senescence (Huffaker and Peterson, 1974). Until the onset of senescence RuBPCase remains a constant percentage of the total soluble protein (Hall et al., 1978; Wittenbach, 1978, Wittenbach et al., 1980). Then, after the onset of senescence, it is degraded preferentially at a rate faster than other proteins, accounting for up to 80% of the loss in total protein (Peoples and Dalling, 1978; Wittenbach, 1978; Friedrich and Huffaker, 1980). During the time when RuBPCase is being

rapidly degraded, labelled amino acids are still being incorporated into it (Peterson and Huffaker, 1975) and other proteins (Tobin and Suttie, 1980). One possible reason that RuBPCase is not synthesized at a rate equal to its degradation is that liberated amino acids move rapidly out of the leaf and are translocated elsewhere in the plant (Storey and Beevers, 1977; Thimann, 1978; Wittenbach, 1979; Wittenbach et al., 1980). That RuBPCase is preferentially degraded is evidence of a controlled proteolytic process. Eventually, though, late in senescence, nonselective enzyme and structural protein lysis follows rapidly after chloroplast envelope degradation (Callow and Woolhouse, 1973; Batt and Woolhouse, 1975; Thomas, 1977).

Enzyme activities exhibit seasonal patterns. Each of the enzymes assayed during the course of senescence in Perilla leaves showed a different pattern of activity (Kannangara and Woolhouse, 1968). Thomas and Stoddart (1980) summarized age-related changes in the isoenzyme complement of 10 enzymes from several plant species. Perhaps the only pattern evident is that enzymes derived from the chloroplastic genome have activity patterns that differ from enzymes derived from the nuclear genome. The activity of several photosynthetic enzymes in Perilla leaves made wholly or partly within the chloroplast began to decline at the time of full leaf expansion, whereas cytoplasm-synthesized enzymes retained a high

activity until later in the season (Batt and Woolhouse, 1975; Woolhouse and Batt, 1978).

The enzyme RuBPCase is of particular interest to crop physiologists because it may regulate phytomass production. The enzyme is composed of eight large, catalytic subunits coded for by the chloroplastic genome and eight small, regulatory subunits coded for by the nuclear genome (Jensen and Bahr, 1977). Thus, the amount and regulation of the enzyme is a result of intracellular interactions. RuBPCase activity on a leaf area or fresh leaf weight basis increases to some maximum at, or near, the time of full leaf expansion and then commences to decline (Peoples and Dalling, 1978; Thomas et al., 1978; Patterson et al., 1980; Wittenbach et al., 1980). The decrease in enzyme activity can be attributed to either a decline in protein amount or enzyme activity. Therefore, a more accurate index of the change in RuBPCase activity is specific activity. RuBPCase specific activity does not fluctuate much until after the onset of senescence when it declines rapidly (Hall et al., 1978; Wittenbach, 1978; Wittenbach et al., 1980). Wittenbach (1979) attributes the loss in specific activity during leaf senescence to a more rapid loss of active sites than of immunological recognition sites, whereas Hall et al. (1978), who observed no decline in the amount of RuBPCase, believe that the decline in specific activity is due to a change in the kinetic form of the enzyme *in vivo*.

Proteases There has been a search for senescence-specific enzymes; i.e., enzymes which increase in activity just prior to or at the time of the onset of leaf senescence. Thomas and Stoddart (1980) present a case for ribonuclease (RNase) as being the key metabolic component that changes with the onset of senescence. They believe that the role of RNase in leaf senescence should be investigated because: (1) the enzyme appears to be active in leaf senescence, (2) it is synthesized de novo (accounting for results with inhibitors), (3) it can be purified and identified simply, and (4) its biochemical function is verifiable. RNase activity has been observed to increase in dark-induced oat leaf disc senescence (Thimann, 1978), but the precise nature of its role in senescence has been questioned (Beevers, 1976; Storey and Beevers, 1977). By far, the greatest attention has been given to the role of proteases in leaf senescence (Anderson and Rowan, 1965; Beevers, 1968; Martin and Thimann, 1972; Peterson and Huffaker, 1975; Dalling et al., 1976; Drivdahl and Thimann, 1977; Feller et al., 1977; Frith et al., 1978; Peoples and Dalling, 1978; Thomas, 1978; Wittenbach, 1978).

Proteolysis is recognized as one of the first major features in leaf senescence (Thimann, 1978; Peoples and Dalling, 1978; Wittenbach et al., 1980). A variety of proteases having an acidic pH optimum have been observed in wheat

(Drivdahl and Thimann, 1977; Frith et al., 1978; Peoples and Dalling, 1978; Peoples et al., 1978; Wittenbach, 1978, 1979), oat (Thimann, 1978), tobacco (Anderson and Rowan, 1965), maize (Feller et al., 1977), pea (Storey and Beevers, 1977) and soybean leaves (Ragster and Chrispeels, 1981b). The use of a wide variety of substrates including casein (Feller et al., 1977; Frith et al., 1978), hemoglobin (Frith et al., 1978) and RuBPCase (Peoples and Dalling, 1978; Wittenbach, 1978, 1979) has made the characterization of protease substrate specificity difficult among studies. Storey and Beevers (1977) reported that proteolytic activity was higher on protein prepared from leaf extract than on bovine serum albumin, casein, or hemoglobin. Some proteases have been shown to have a high affinity for RuBPCase (Woolhouse, 1967; Wittenbach, 1978), which agrees with the preferential degradation of Fraction-I protein mentioned earlier. A problem arises because proteases are believed to be synthesized on cytoplasmic 80s ribosomes (Peterson and Huffaker, 1975; Thimann, 1978), whereas the substrate is located in the chloroplast. Although the mechanism is unknown, proteases do enter the chloroplast (Choe and Thimann, 1975), but the pH of the stroma is too basic for the acidic protease to have appreciable activity. Dalling et al. (1976) postulated that proteases may be active in localized zones of low pH within the chloroplasts, whereas Wittenbach (1978) speculated that the high selectivity of the

protease toward RuBPCase is due to either compartmentalization or a high affinity for the RuBPCase.

The role of proteases in the initiation and continuation of proteolysis during leaf senescence is moot. In maize, foliar senescence symptoms are paralleled by decreases in exopeptidase, protein content and by an increase in endopeptidase activity (Feller et al., 1977). Similar findings were observed in wheat, with the exopeptidases, aminopeptidase and carboxypeptidase decreasing in activity during nitrogen mobilization from the leaf, while endopeptidase activity increased (Feller and Erismann, 1978). Thus, an endopeptidase seems to be active in leaf senescence in maize and wheat leaves. The loss of RuBPCase is correlated negatively with the appearance of an unspecified protease in barley (Peterson and Huffaker, 1975) and soybean leaves (Wittenbach et al., 1980). But the role of endopeptidases in soybean leaf senescence is likely to be different than in cereal leaf senescence. Ragster and Chrispeels (1981b) showed that three acidic endopeptidases were present throughout soybean leaf development with activities decreasing during senescence. Further proof that legume and cereal proteases may differ is that Feller (1979) reported a constant exo- and endopeptidase activity over the life of field bean leaves. This led Feller to conclude that "the large increase in neutral endopeptidase observed in leaves of maturing cereals could not be found in

bean leaves" and that "a different regulation of nitrogen mobilization and of proteolytic activities appears reasonable between nitrogen metabolism of cereals and legumes."

A close relationship between RuBPCase activity and photosynthesis should be expected in C_3 species. Ideally, as RuBPCase amount or activity declines, a consequent decline in photosynthesis should occur. In cereals, the decline in photosynthesis during leaf senescence is preceded by a decline in total soluble protein (Wittenbach, 1979; Friedrich and Huffaker, 1980), RuBPCase content (Friedrich and Huffaker, 1980) and RuBPCase specific activity (Hall et al., 1978; Wittenbach, 1979; Friedrich and Huffaker, 1980). The temporal and causal relationship between RuBPCase and photosynthesis is less clear in soybean leaves. In depodded soybean plants, leaf photosynthesis declined, but *in vitro* RuBPCase activity on a leaf area basis remained high (Mondal et al., 1978). The ontogenetic decline in leaf photosynthesis in field-grown soybean plants could not be accounted for by a decline in RuBPCase activity or level (Wittenbach et al., 1980). Therefore, other factors may be regulating photosynthetic rate in soybean leaves.

Nucleic acids

One of the most characteristic and peculiar features of plant cells is the spatially separated yet functionally integrated chloroplast and nuclear genomes. Each genome operates

independently and in one very important case--the synthesis of RuBPCase--both genomes are required. But the synthesis of RuBPCase indicates that the operation and regulation of the two genomes is dissimilar and uncoordinated. The rate of synthesis of the regulatory, small subunit of RuBPCase, which occurs on cytoplasmic ribosomes, is faster than the translation of the large subunit in the chloroplast (Feierabend and Wildner, 1978; Barraclough and Ellis, 1979). Furthermore, during early leaf development, the formation of the large subunit seems to be the rate limiting step in the synthesis of RuBPCase (Feierabend and Wildner, 1978). It is believed that, later in leaf development, the chloroplast genome actually becomes inactive (Batt and Woolhouse, 1975; Woolhouse and Batt, 1976; Brady et al., 1971), which has led Thomas and Stoddart (1980) to suggest that "the chloroplast genome seems to exert an influence over senescence largely through its inactivity."

The characteristic decline in protein content during leaf senescence is coincident with a decline in RNA content (Beevers, 1976). Work by Osborne (1962) tends to show that, rather than an increase in rate of degradation, RNA synthesis is halted because of a failure of DNA to provide an effective template for transcription, leading to eventual leaf senescence. Other results support Osborne's observation that RNA synthesis becomes impaired. By measuring the loss of

polyribosomes (Srivastava and Arglebe, 1967) and/or free ribosomes (Srivastava and Arglebe, 1967; Eilam et al., 1971; Callow et al., 1972), investigators have provided evidence that rate of RNA synthesis declines.

However, the onset of senescence also has been attributed to the production of chromatin-associated nucleases that degrade newly synthesized RNA (Osborne, 1962; Srivastava, 1968; Dyer and Osborne, 1971; Trewavas, 1970; Pollock and Lloyd, 1978). These nucleases are probably specific for certain types of nucleic acid because t-RNA is relatively stable during senescence (Dyer and Osborne, 1971). Nucleic acid degradation during senescence has been clearly shown to differ among species. No RNA is present in senescing leaves of Xanthium pennsylvanicum (L.), whereas both cytoplasmic and chloroplastic fractions are found in yellow leaves of Vicia faba (L.) and tobacco (Dyer and Osborne, 1971).

Regulation of Leaf Senescence

That there is an ordered sequence of events occurring during leaf ontogeny is indicative that leaf senescence is a preprogrammed and regulated process. As yet, the internal control mechanism for this process has not been elucidated. Extensive studies have been made using detached leaves or leaf discs to determine the mechanism by which senescence is controlled. Although excised leaf material is a convenient

experimental material, it suffers from some serious drawbacks, particularly if the detached material is artificially induced to senesce. Furthermore, excised or detached leaf material is chosen often without regard to plant or leaf age, or to leaf position on the plant. More potential problems arise because detachment can induce wound responses and, most obviously, severs translocation and communication with the rest of the plant. Dark-induced senescence further complicates interpretation of the senescence program because light is required for some essential metabolic processes; e.g., chlorophyll synthesis (Salisbury and Ross, 1979) and RuBPCase turnover (Dockerty et al., 1977; Nishimura and Akazawa, 1978). Thus, if the progression of dark-induced senescence is measured by the loss of chlorophyll or protein, then an inaccurate assessment of the metabolic sequence of senescence is probable. Nevertheless, it would be inappropriate to ignore totally the work done with excised or detached leaf material. As Beevers (1976) wrote,

> Observations that senescence of detached leaves can be controlled by exogenous application of growth regulators indicate that leaf senescence in the intact plant may be regulated by the balance of endogenous growth regulators.

Light

As mentioned in the previous paragraph, light does have a direct influence on metabolic functions. It has been implicated also in the retardation of senescence, although the

mechanism by which it exerts this effect is still a matter of conjecture. In excised leaf material, the effect of light has been attributed to the products of photosynthesis (Goldwaite and Laetsch, 1967; Malik and Thimann, 1980), retardation of chloroplast degradation (Haber et al., 1969), and to the photomorphogenic effects of phytochrome (Sugiura, 1963; DeGreef et al., 1971; Biswal and Sharma, 1976).

Plant growth regulators

Much attention has been devoted to studying the effect of plant growth regulators on leaf senescence (cf. Nooden and Leopold, 1980). By far, the cytokinins have been the most investigated plant hormone. Beginning with Richmond and Lang's (1957) finding that kinetin delayed senescence in Xanthium leaf discs, the search to uncover the mode of action of cytokinins has been widespread. The most prevalent belief is that, at least in excised leaf tissue, the cytokinins retard protein degradation (Kurashi, 1968; Shiboaka and Thimann, 1970; Martin and Thimann, 1972; Peterson and Huffaker, 1975; Wittenbach, 1978). Other senescence delaying effects attributed to the cytokinins include increased RNA synthesis (Osborne, 1962; Trewavas, 1970), increased protein synthesis (Atkin and Srivastava, 1970), increased starch mobilization (Berridge and Ralph, 1971), and increased "general" metabolism (Adepipe and Fletcher, 1970). Cytokinins have been less effective in delaying leaf senescence

in intact plants (Woolhouse, 1978). No effect on protein metabolism was observed in wheat leaves painted with kinetin (Hall et al., 1978). Slightly more optimistic results were obtained by a foliar spraying of cytokinins onto soybean leaves, where leaf senescence was clearly delayed but not prevented (Lindoo and Nooden, 1978).

Abscisic acid (ABA) is another plant hormone believed to play a role in leaf senescence. Early work with ABA showed that RNA levels were reduced in leaf material floating on ABA solutions (Trewavas, 1970). Foliar spraying of ABA on intact soybean leaves significantly accelerated leaf yellowing (Lindoo and Nooden, 1978). And a natural increase in ABA-like substances has been observed in near senescent (Lindoo and Nooden, 1978) and yellowing (Samet and Sinclair, 1980) soybean leaves. Although Lindoo and Nooden concluded that ABA could be a possible cause of monocarpic senescence, Samet and Sinclair (1980) contended that the ABA rise in soybean leaves was an effect rather than the cause of leaf senescence.

Other plant growth regulators have been implicated in the leaf senescence process. A substance in Artemisia absinthium L., identified as (-) methyl jasmonate, has been shown to have a much stronger senescence-promoting effect than ABA (Ueda and Kato, 1980). The polyamines, putrescine, spermidine and spermine prevent the loss of chlorophyll

normally associated with the senescence of excised leaf tissue maintained in darkness (Cohen et al., 1979). In a series of papers, ethylene was shown to play a major role throughout the senescence process of dark-induced tobacco leaf discs (Aharoni and Lieberman, 1979; Aharoni et al., 1979a, b).

Although plant growth regulators seem to have a substantial effect on excised leaf material, their effect on intact plants has not yet been proved. Thomas and Stoddart (1980) believe that,

> On present evidence neither a decline in endogenous levels of any of the currently known senescence-retarding hormones, nor increases in a senescence promotor, seems to be the primary event in the induction of the senescence program.

Summary and Conclusions

Physiologically, the most logical reference point for the onset of senescence is the time when photosynthesis begins to decline. Most metabolic substances and processes, including chlorophyll, protein and photosynthesis, increase during early stages of plant and leaf growth, reach a maximum, and decline. The focus of many senescence-related studies has been to elucidate the metabolic and temporal sequence leading up to and following the onset of senescence. Photorespiration, mitochondrial respiration and leaf diffusive resistance seem not to be principal factors in leaf senescence.

On the other hand, chlorophyll and protein seem to be closely related with the decline in photosynthesis. However, in two species, soybean and fescue, chlorophyll metabolism was separated from the decline in photosynthesis. Changes in protein amount and enzyme activities, notably RuBPCase, in nearly all cases studied are closely related to changes in photosynthesis. This has led to investigations focused on identifying and characterizing senescence-initiating enzymes, particularly proteases. To date, no universal or particular scheme has been laid down to account for the sequence of events leading up to senescence.

A precise understanding of the progression of events in senescence may be lacking partly because of (1) the lack of a widely accepted definition of senescence, (2) the diversity of indices used to measure senescence, and (3) the various methods used to induce senescence. Moreover, clear understanding of the events in leaf senescence is further hampered in those studies where leaves from an intact plant are selected on the basis of a specific node relative to the growing apex, thus obscuring the characterization of individual leaf ontogeny and senescence because the age of the sampled leaf does not change until plant elongation and leaf emergence ceases. In wheat flag leaves, in which leaf development is easy to follow, the sequence of senescence-related events is understood better than in soybean leaves, in which individual

leaf ontogeny is more difficult to follow (Wittenbach, 1979; Wittenbach et al., 1980). The study of ontogenetic metabolic changes of individual soybean leaves is clearly needed to advance the understanding of leaf and plant senescence in legumes.

PART I. COMPARISONS BETWEEN LEAVES OF SIMILAR PLANT ONTOGENY

EXPERIMENTAL PROCEDURE

Plant Material and Culture

The plant material was grown in two north-south oriented wooden bins, measuring 0.46 m x 0.46 m x 6.10 m, located outdoors between the east and center Agronomy greenhouses. The bins were situated 1.07 m apart and were no closer than 1.52 m to any exterior greenhouse wall. They were filled with a mixture containing 2 parts soil:1 part peat:1 part sand. Fertilizer, prepared by dissolving 240 g KH_2PO_4 and 80 g K_2SO_4 in 2 liters of water, was applied to each bin in trenches approximately 11 cm from the inside bin wall and approximately 18 cm below the soil surface.

'Amsoy-71' soybean seeds were planted on 25 May 1979 at the rate of 12 seeds per 30 cm in a single row along the center of each bin. On 6 June 1979 the plants were thinned to 6 plants per 30 cm based on uniformity of growth and development. The plants were prevented from lodging by allowing them to grow through a nylon netting supported approximately 80 cm from the soil surface.

The bins were maintained weed-free by hand weeding. To prevent water stress, plants were irrigated on a regular basis. Insects were controlled by the application of malathion (0,0-dimethyl dithiophosphate of diethyl mercaptosuccinate) to the plants and the area surrounding the bins.

Spider mites (Tetranychus telarius L.) were controlled by periodic applications of Plictran (tricyclohexyltin hydroxide).

Sampling Protocol and Procedures

Plant selection and sampling

I sampled leaves at nodes 12 and 15 (first trifoliolate leaf node = 1) because (1) the leaves at these nodes developed during the reproductive period of plant ontogeny and (2) the nodes were as spatially separated on the stem as was possible to have leaves, which developed during reproduction, large enough to fit into the gas-exchange leaf chamber.

On 18 July 1979, the center 100 plants in each bin were numbered. Ten unnumbered plants at each end of each bin served as unsampled border plants. The 200 numbered plants were surveyed on 19 July 1979, 55 days after planting, to determine their stage of growth and development. Two groups of plants were discernible: plants in which the 12th leaf was about to emerge and plants in which the 13th leaf was about to emerge. I chose to use the former group of plants to measure the leaves of node 12, whereas the latter group was to be used to measure leaves at node 15. There were several reasons for this decision: (1) to minimize variability owing to leaf emergence dates, (2) to assure that leaflet size at the 15th node was large enough to fit into the

gas-exchange leaf chamber (leaflet area at older nodes is smaller than at middle-aged nodes) and (3) to be sure that there would be enough plants to sample leaves at each node over the season.

Leaves at node 12 were sampled on 10 days over the season (from 30 July to 11 September 1979), whereas leaves at node 15 were measured on 8 days (from 4 August to 13 September 1979). The sampling commenced when the expanding leaflets at a given node were large enough to fit into the gas-exchange leaf chamber and ceased when the leaves began to abscise. On a given sampling day, one leaf from each of four plants was measured. Leaves from node 12 were never measured on the same day as leaves from 15. The same plant never had both its node-12 and node-15 leaves sampled. Thus, there was a total of 72 plants sampled, 40 for node-12 and 32 for node 15 leaves.

The daily general sample protocol is outlined in Figure 1. On the day before sampling, leaves from four randomly chosen plants of the group to be sampled (node-12 or node-15) were inspected for damage and, if found to be healthy, were tagged. On the sampling day, diffusive resistance, carbon dioxide exchange rate (CER), and dark respiration (Rn) were measured on the terminal leaflet of the selected leaf, starting at 10:00 h CDT, <u>in situ</u>. Then the leaflets were harvested, brought into the laboratory, which was in the green-

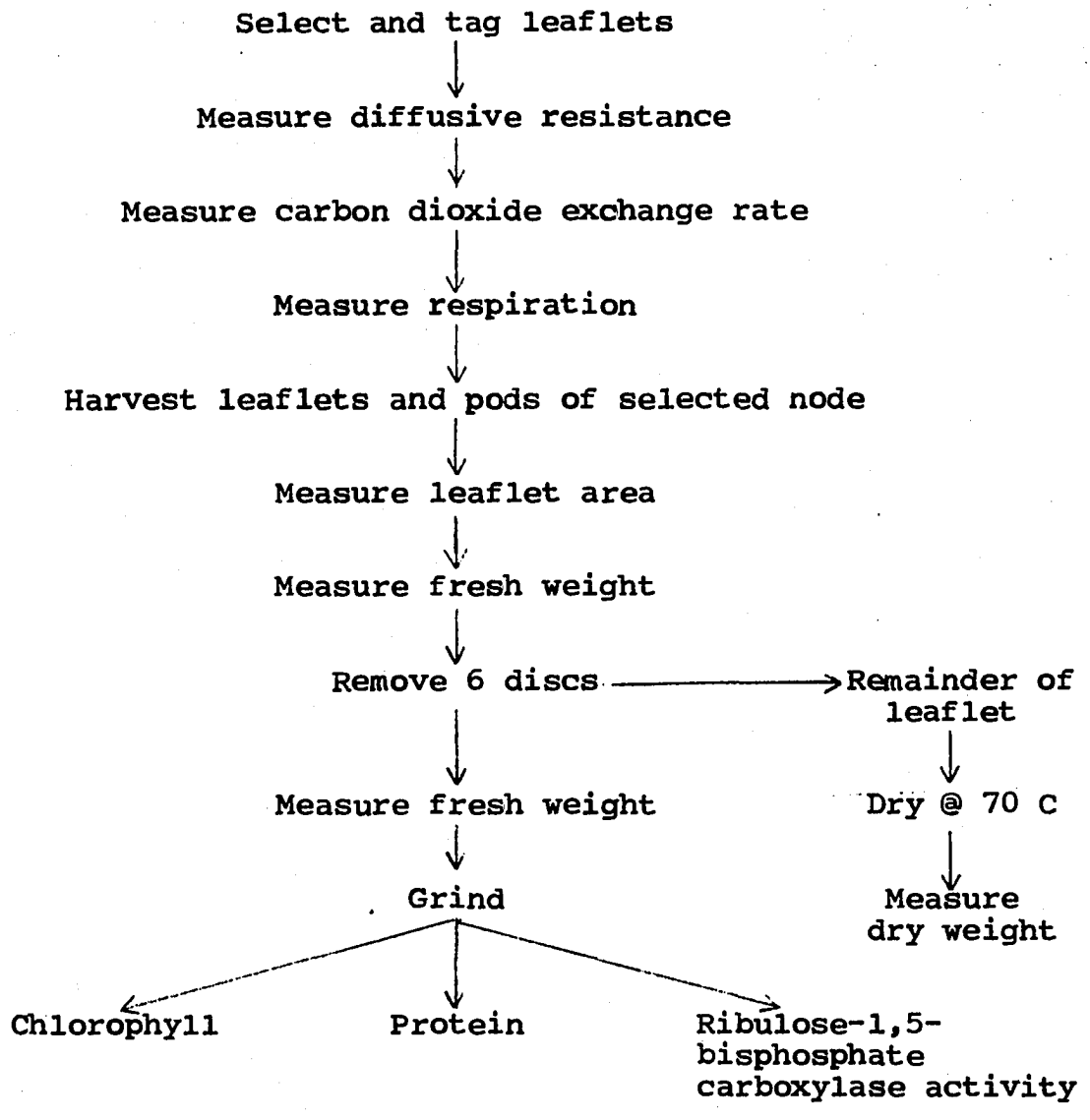


Figure 1. General sampling procedure--1980

house headhouse, and analyzed for biochemical and physiological parameters as described later.

Plant Measurements

Diffusive resistance

The instrument used to measure diffusive resistance was a LiCor Model LI-65 Autoporometer (LiCor Inc., Lincoln, Nebraska) equipped with a LI-20s LiCl sensor. The instrument was calibrated according to the directions supplied by the manufacturer. Calibrations were performed before the experiment began and several times throughout the sampling period. Four measurements, two adaxial and two abaxial, were taken on each leaflet. The measurements were alternated between the adaxial and abaxial surfaces on both lateral halves (left and right) of the leaflet. To minimize possible diurnal effects of water status or leaf temperature, the diffusive resistance measurements were completed on all four plants (20 min) before CER was measured on any one.

Gas exchange

CER was estimated by using a Beckman (Beckman Instruments Inc., Fullerton, California) Model 865 infrared gas analyzer (IRGA) in an open-system, schematically diagrammed in Figure 2. In this particular system, carbon dioxide is adjusted to a desired concentration by first removing it from the incoming atmospheric air and then introducing pressurized carbon

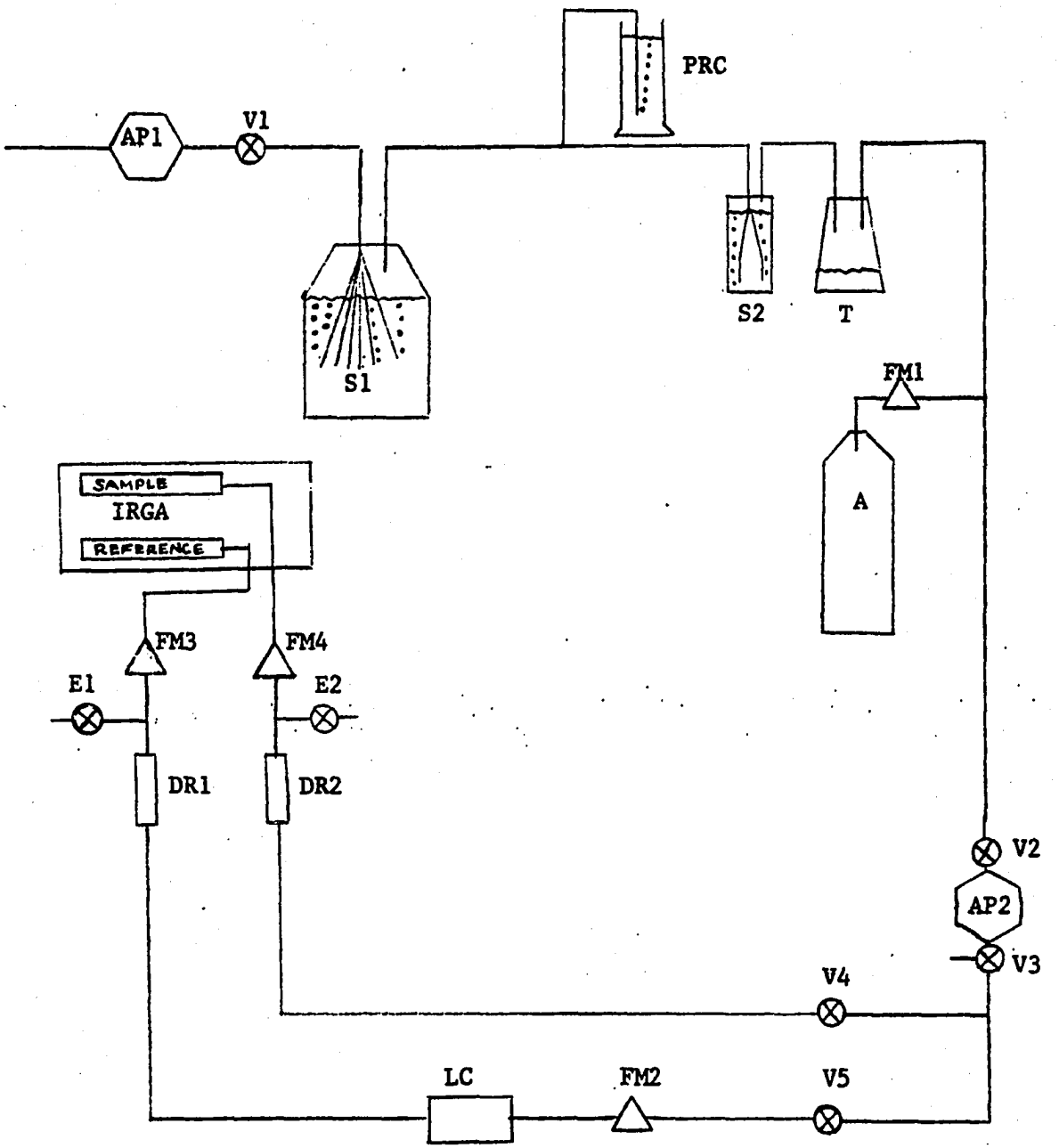


Figure 2. Schematic diagram of the gas exchange system (Symbols used: AP1,AP2, air pumps; S1, 6N KOH; S2, 6N NaOH: PRC, pressure relief column; V1,V2, V3,V4,V5, gas valves; T, cold water trap; FM1,FM2, FM3,FM4, flowmeters; A, 5% CO_2 in air; LC, leaf chamber; DR1,DR2, drying columns; E1,E2, exhaust valves; IRGA, infrared gas analyzer)

dioxide at the desired concentration. The gas stream is then divided into two lines, one to the leaf chamber and the other to the sample cell of the IRGA. The gas from the leaf chamber is directed into the reference cell of the IRGA, where the carbon dioxide concentration is compared with the carbon dioxide concentration in the sample cell. To obtain a positive reading from the IRGA, the gas returning from the leaf chamber, which has a lower CO_2 concentration than the reference gas, must enter the reference cell of the IRGA.

The specifics of the system can be explained most lucidly by following the gas pathway shown in Figure 2. Unless otherwise specified, the gas flowed through 0.64 cm (inside diameter) Tygon (Norton, Akron, Ohio) R-3603 tubing. Atmospheric air was drawn into the laboratory by an air pump (Duraire diaphram-type pressure pump), AP1, and then forced through six plastic gas dispersion tubes immersed in 12 l of 6N KOH (S1) at 4 C. The gas then flowed through two glass gas dispersion tubes immersed in a second solution (S2) containing 750 ml of 5N NaOH at 4 C. The combined effect of these two solutions reduced the CO_2 concentration in the gas to less than 15 $\mu l \cdot l^{-1}$. A slight positive pressure was maintained between the two solutions by adjusting the needle valve V1 so that a slow, steady stream of bubbles appeared in the pressure relief column, PRC. The pressure relief column was constructed of a water-filled 1 liter plastic graduated

cylinder with a glass tube attached to the inner wall. Gas flowing through S2 passed through a cold water trap (T) which collected any NaOH that may have accumulated and moved in the Tygon tubing. Air containing 5% CO_2 was metered from a pressurized tank (Matheson, Joliet, Illinois), A, into the CO_2-stabilized gas through a flowmeter, FM1, equipped with a high accuracy needle valve. The resulting gas mixture then entered a second air pump, AP2 (same design as AP1). An intake vacuum of 103 KPa (15 $lb \cdot in^{-2}$) and an exhaust pressure of 69 KPa (10 $lb \cdot in^{-2}$) were maintained by a needle valve (V2) on the intake side and a pressure relief valve (V3) on the exhaust side of the air pump. The gas stream was then divided into two lines, one to the IRGA sample cell and the other to the leaf chamber (LC). The gas flow to the IRGA was adjusted to 120 $l \cdot h^{-1}$ by needle valve V4, while the gas flow to the leaf chamber was adjusted to 180 $l \cdot h^{-1}$ by needle valve V5. The gas stream to the leaf chamber travelled approximately 20 m from the laboratory in aluminum-foil-covered, foam-insulated, H_2O-jacketed, copper tubing to the plant growth site outdoors. The end of the copper tubing was attached, via Tygon tubing, to a needle-valve-equipped flowmeter (FM2), which was adjusted to deliver a flow rate of 120 $l \cdot h^{-1}$ to the leaf chamber. From FM2 to the leaf chamber, the gas flowed through aluminum-foil-covered, foam-insulated Tygon tubing. The gas returning from the leaf chamber to the IRGA in the laboratory followed the reverse

route of the gas to the leaf chamber. Before entering the IRGA cells, both the leaf chamber gas and the reference gas were dehumidified in Drierite ($CaSO_4$) columns (DR1, DR2). The flow rates to the IRGA cells were adjusted to 96 $l \cdot h^{-1}$ (FM3 and FM4) by exhausting excess gas via needle valves E1 and E2.

The IRGA was calibrated by using two primary-grade standard gases (Matheson, Joliet, Illinois). The zero adjustment was made by flowing 324 $\mu l \cdot l^{-1}$ CO_2 in N_2 through both the sample and reference cells. The upscale was set to 73 by flowing 251 $\mu l \cdot l^{-1}$ CO_2 in N_2 through the reference cell and 324 $\mu l \cdot l^{-1}$ CO_2 in N_2 through the sample cell. After the IRGA was calibrated, the incoming, CO_2-depleted gas was adjusted to 316 $\pm$ 5 $\mu l \cdot l^{-1}$ CO_2 and checked periodically throughout the day.

The plexiglass leaf chamber (Figure 3) was designed to sample a 11.4 cm^2 portion of the leaflet. The center of the leaflet was positioned in the middle of the chamber, and the chamber halves were tightened by two wing nuts (not illustrated). A closed-cell foam rubber gasket on the upper and lower chamber halves sealed the leaflet and chamber surfaces and prevented the exchange of chamber and atmospheric gases. Gas entered both the upper and lower halves of the chamber via 10 intake ports and exited via 10 exhaust ports. Temperature was controlled by adjusting the flow of cold tap water through the water jackets.

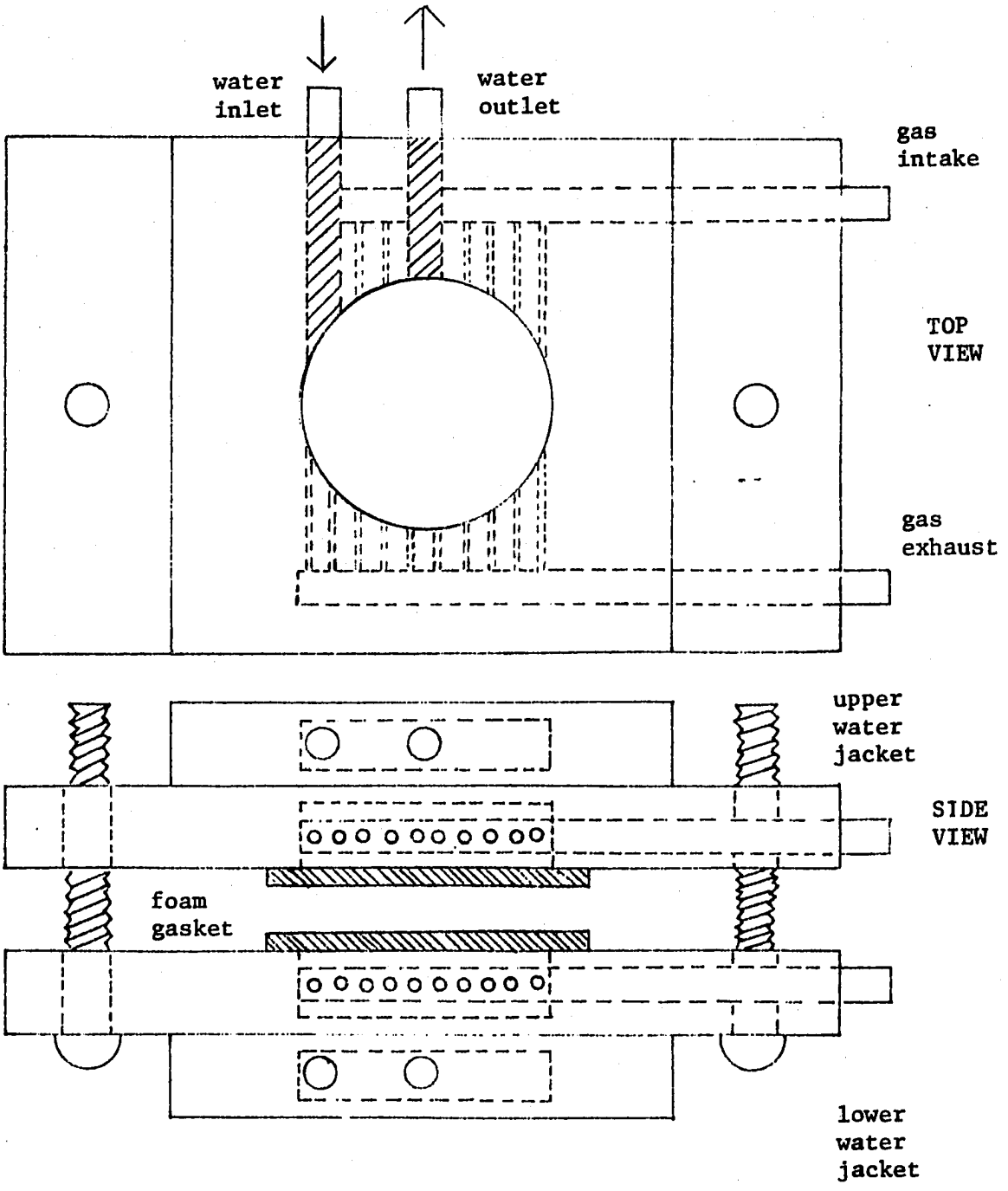


Figure 3. Top and side view of leaf chamber; scale 1:1

CER was measured in the following manner. After calibrating the IRGA and adjusting the CO_2 concentration of the gas, the flow rate to the leaf chamber was adjusted to 120 $1 \cdot h^{-1}$ at FM2. The leaf chamber was clamped over the center part of the terminal leaflet and positioned normal to the sun. On partly cloudly or slightly overcast days, supplemental irradiance was supplied by a tungsten filament light source, which provided 2000 mol quanta $s^{-1}m^{-2}$ of PPFD. The water flow to the leaf chamber was adjusted to maintain a leaf temperature of 27 ± 3 C, as measured by a copper-constantan thermocouple appressed against the abaxial surface of the leaflet in the leaf chamber. The CO_2 concentration was recorded after a steady state of CER was reached, which was usually about 10 min after the measurement began. CER was calculated from the following formula:

$$CER = \left(\frac{\mu l\ CO_2}{1\ gas}\right)(FR)\left(\frac{273}{T}\right)\left(\frac{1000\ n\ mol}{22.4\ \mu l}\right)\left(\frac{1}{A}\right)$$

where:

FR = flow rate (0.0333 $1 \cdot s^{-1}$)

A = exposed leaf area (11.4 cm^2)

T = leaf temperature (K)

Dark respiration (Rn) was measured immediately after CER. With the leaf still in the chamber, the chamber was covered with a black plastic bag, the gas flow to the chamber was reduced to 60 $1 \cdot h^{-1}$ at FM2, and the cold tap water flow in the water jackets was adjusted to obtain a leaf temperature of

23 ± 2 C. Because the gas flow rate to the leaf chamber was reduced, the flow rate to the IRGA was also reduced. Therefore, the flow rates to both IRGA cells were adjusted to 48 $l \cdot h^{-1}$ at flowmeters FM3 and FM4. A steady state of gas exchange was attained, usually in about 25-30 min. Respiration rate was calculated by using the same formula used for CER, except the flowrate term was 0.167 $l \cdot s^{-1}$.

Plant and leaflet data

The leaf number from the plant apex was recorded while the gas exchange measurements were being taken. After the gas exchange measurements were completed for the four plants, the terminal leaflets were removed, individually placed in moist plastic bags, and brought into the laboratory.

The area of the terminal leaflet was measured on a LI-COR (LiCor Inc., Lincoln, Nebraska) Model LI-3000 leaf area meter equipped with a transparent conveyor belt accessory. Following the measurement of leaflet area, the leaflet was weighed, after which six 1.42-cm^2 discs were removed from the interveinal portion of the leaflet, by use of a number 10 cork borer, and weighed. The remaining portion of the leaflet was placed in a labelled test tube and dried at 70 C for at least 24 h. Specific fresh weight (SFW) was calculated by dividing the leaflet's fresh weight by its area. Specific leaf weight (SLW) was determined by dividing the dry weight of the perforated leaflet by its respective area (total leaflet area

minus disc area).

Pods, if present at the leaf node, were removed when the leaflet was harvested, placed in a labelled test tube and dried at 70 C for at least 24 h.

Biochemical and Analytical Assays

Tissue preparation

The leaflet tissue was prepared for biochemical measurements by placing the six leaf discs into a cold (4 C), motorized, Ten Broeck, ground-glass tissue homogenizer. Then 10 ml of cold, CO_2-free, grinding buffer, which contained 100 mM Tris (pH 8.6), 10 mM $MgCl_2$, 0.25 mM EDTA, 5 mM dithiothreitol and 5 mM isoascorbate, was added. The tissue was ground for approximately 2 min, and the resulting extract was poured into a test tube and stored at 4 C until used.

Chlorophyll

Two 1.0 ml aliquots from the extract were each mixed with 4.0 ml of acetone in a centrifuge tube. The tube was then stoppered and stored overnight at 5 C in a dark refrigerator. The following day, the samples were centrifuged for 10 min at 3,000 x g and the resulting supernatants were spectrophotometrically assayed according to the method of Arnon (1949).

Protein

Two 50 µl aliquots from the extract were placed into separate test tubes and 2.95 ml of deionized, distilled water were added to each. The test tubes were then stoppered and stored overnight at 5 C in a dark refrigerator. The protein in each test tube was precipitated with Na deoxycholate and trichloroacetic acid according to the method of Bensodoun and Weinstein (1976). The precipitate was solubilized in 0.3 N NaOH and assayed by the modified Lowry technique of Miller (1959). Bovine serum albumin was used as a standard.

RuBPCase activity

Ribulose bisphosphate carboxylase (RuBPCase) (E.C. 4.1.1.39) activity was estimated by the incorporation of $^{14}CO_2$ into acid-stable products.

Solutions of ribulose 1,5-bisphosphate (RuBP) (Sigma, St. Louis, Missouri) were prepared within three days of use and stored at -20 C. Radioactive $NaHCO_3$ (Amersham, Arlington Heights, Illinois) was prepared to 200 mM with a specific activity of 8.41 Bq·nmol^{-1} (0.22 nCi·nmol^{-1}) and stored at 5 C.

Duplicate assays were performed on each extract. Each assay was carried out in a 20-ml liquid-scintillation vial which had been purged with N_2 gas, and to which was added 430 µl of CO_2-free buffer containing 100 mM Tris (pH 8.2), 20 mM $MgCl_2$ and 5 mM dithiothreitol. The vial was then capped with a serum stopper, and 50 µl of 200 mM (^{14}C) $NaHCO_3$

(8.14 Bq·nmol^{-1}) and 10 µl of the extract were injected into the vial. The resulting mixture was incubated for 10 min at room temperature to allow for activation of the RuBPCase. After 10 min, the reaction was initiated by injecting 10 µl of 20 mM RuBP into the vial; the reaction was terminated after 60 s by injecting 100 µl of 2N HCl. The serum stopper was removed in a fume hood and after 15 min the vial was placed in a 90 C oven in the fume hood. When the residue had dried, the vial was removed from the oven and 1.0 ml H_2O plus 3 drops of ethanolamine were added to each vial. The vial was swirled before 12 ml of Handifluor (Mallinckrodt, Paris, Kentucky) liquid-scintillation cocktail was added. The vial was capped, vortexed, and the radioactivity was counted in a scintillation spectrometer (Packard Tri-Carb).

The 0.3:1.0 channels ratio method was used to determine counting efficiency. A quench correction curve was constructed using commercially prepared, sealed, quenched standards and then calculating the curve by linear regression.

Activity was calculated according to the following formula:

$$\text{Activity} = \left(\frac{\text{CPM}}{\text{efficiency}}\right)\left(\frac{1\ \text{Bq}}{60\ \text{dpm}}\right)\left(\frac{\text{n mol}}{8.14\ \text{Bq}}\right)\left(\frac{1}{\text{min}}\right)$$

Preliminary experiments showed that the assay was linear with both time and amount of extract.

RESULTS AND DISCUSSION

Mean squares from the analyses of variance for the measured parameters are in Tables A1, A3 and A4 in the Appendix. The means for each day and appropriate least significant differences (L.S.D.) are listed in Table A2 in the Appendix.

In the following sections, particular attention is given to how a parameter changes over the season and to how the seasonal trend of that parameter differs between leaves at the 12th and 15th nodes.

Physical Parameters

Leaflet number and leaflet area

The leaf number from the apex represents the position of the sampled leaf relative to the growth of the plant. Thus, the time at which leaf production ceases coincides with the time when leaf number stops increasing. The seasonal changes in leaf number for node 12 and node 15 are presented in Figure 4A. It can be seen that sampling commenced at both nodes when the leaves were approximately the third from the apex. The group of plants from which leaves at node-12 (node-12 plants) were sampled seemed to continue to grow throughout the season, whereas growth apparently stopped after day 74 in those plants from which leaves at node-15 (node-15 plants) were sampled. The continuation of plant elongation in node-12 plants is biologically unlikely because soybean plants are

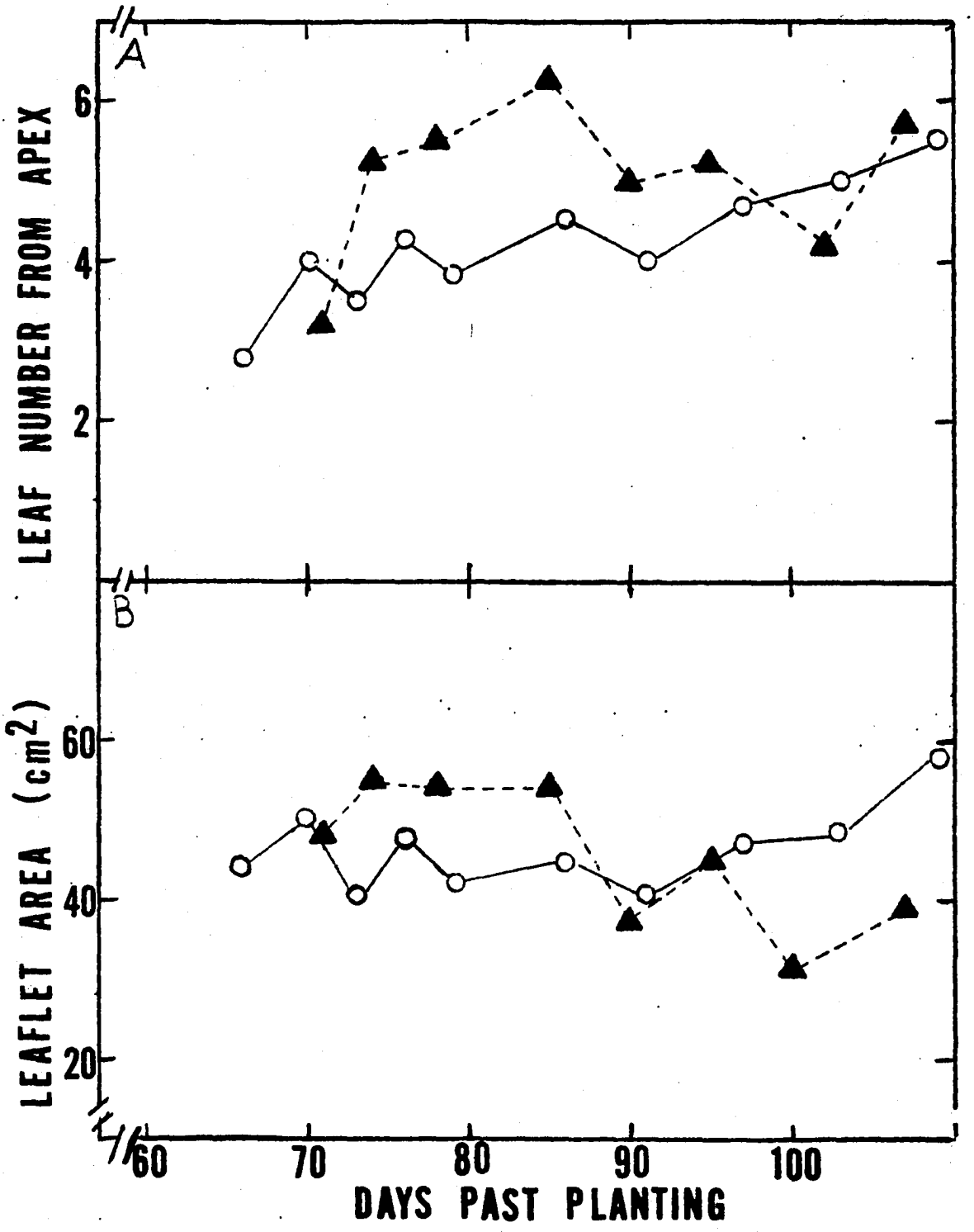


Figure 4. Seasonal trends of leaf number (A) and terminal leaflet area (B) for nodes 12 (O) and 15 (▲)--1979

known to stop growth during the late reproductive period. Because the rate of growth displayed in Figure 4A for node-12 plants is exceptionally low, about one leaf every eight days, and according to least significant difference (L.S.D.) tests, in which no significant day-to-day differences were detected after day 70, it is most probable that plant growth ceased soon after day 70 in node-12 plants. The apparent increase in leaf number for node-12, then, represents sampling variability only.

A peculiarity seen in Figure 4A is that the sampled leaves from the node-15 plants were lower on the plant than the sampled leaves from the node-12 plants. The probable cause for this irregularity is the method by which the plants were selected for sampling. On 19 July, 11 days before sampling began, all plants were inspected and then divided into two groups according to their growth rate. Plants in which the 11th leaf (node) was emerging were placed in one group (node-12 plants), plants in which the 12th leaf was emerging were placed in a second group (node-15 plants). The node-15 plants were faster growing than node-12 plants, and thus, the leaves at node-15 were lower on their respective plants than the leaves at node-12 on the slower growing node-12 plants. By the time plant elongation stopped, node-15 plants had about 20 nodes, whereas node-12 plants had only about 16 nodes.

The seasonal changes in terminal leaflet area of node-12 and node-15 leaves are shown in Figure 4B. No significant increase in leaflet area at node-12 and node-15 was observed at the beginning of the sampling period. Thus, the leaves had reached full expansion by the time sampling had begun. Leaflet area for node-12 plants had less variability over the season than it did for node-15 plants. And the seasonal means for leaflet area were similar for leaves at both nodes.

Specific leaf fresh weight

The seasonal patterns of specific leaf fresh weight (SFW) are presented in Figure 5A. Except for the increase between days 71 and 74 in leaves at node-15, there was little variation in SFW over the season for leaves at either node. SFW was consistently greater throughout the season in leaves at node-15 than at node-12. Inasmuch as SFW represents both the water status and dry weight of a leaf, it may poorly indicate changes in the amount of leaf photosynthetic tissue. A more accurate index of fluctuation in leaf tissue mass is specific leaf dry weight, the leaf dry weight per unit leaf area.

Specific leaf dry weight

Correlations between specific leaf dry weight (SLW) and SFW indicated that SLW accounts for 56% ($r=0.75$) and 69% ($r=0.83$) of the variation in SFW in node-12 and node-15 leaves,

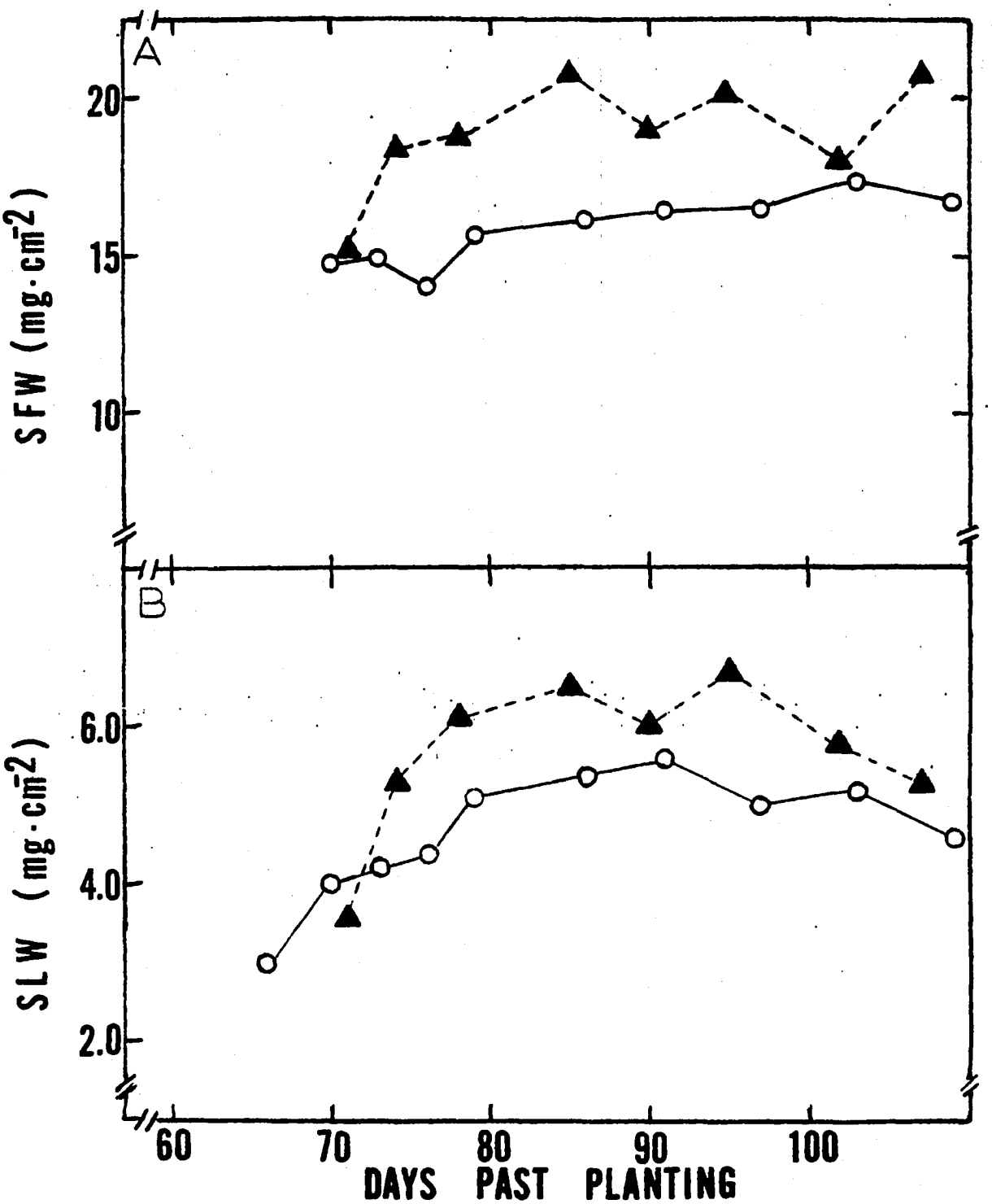


Figure 5. Seasonal trends of specific leaf fresh weight (SLW) (A) and specific leaf dry weight (SLW) (B) for nodes 12 (O) and 15 (▲)--1979

respectively. Higher correlations may have occurred if SLW and SFW had been measured on identical leaf material. SFW was calculated on the whole leaflet, whereas SLW was determined from the same leaflet after discs had been removed. Because the discs were removed from the interveinal portion of the leaflet blade, SLW was affected by the unequal percentage among the leaflets of veinal tissue remaining in the perforated leaflet blades.

There was a significant increase in SLW in leaves at both nodes between their respective first two sampling days (Figure 5B). In node-15 leaves, SLW was similar from day 78 to day 95, after which a significant decline began. For node-12 plants, SLW continued to increase slowly after the second sampling day, reached a maximum by day 79, after which no significant day-to-day differences were detected until after day 102.

Pod dry weight

A rapid, linear increase in pod dry weight at both nodes began on day 70 (Figure 6). Thus, leaves at both nodes were sampled during seed filling. The rate of pod filling differed between the two nodes with node-12 having not only a greater pod-filling rate, but also a greater final pod dry weight than node-15.

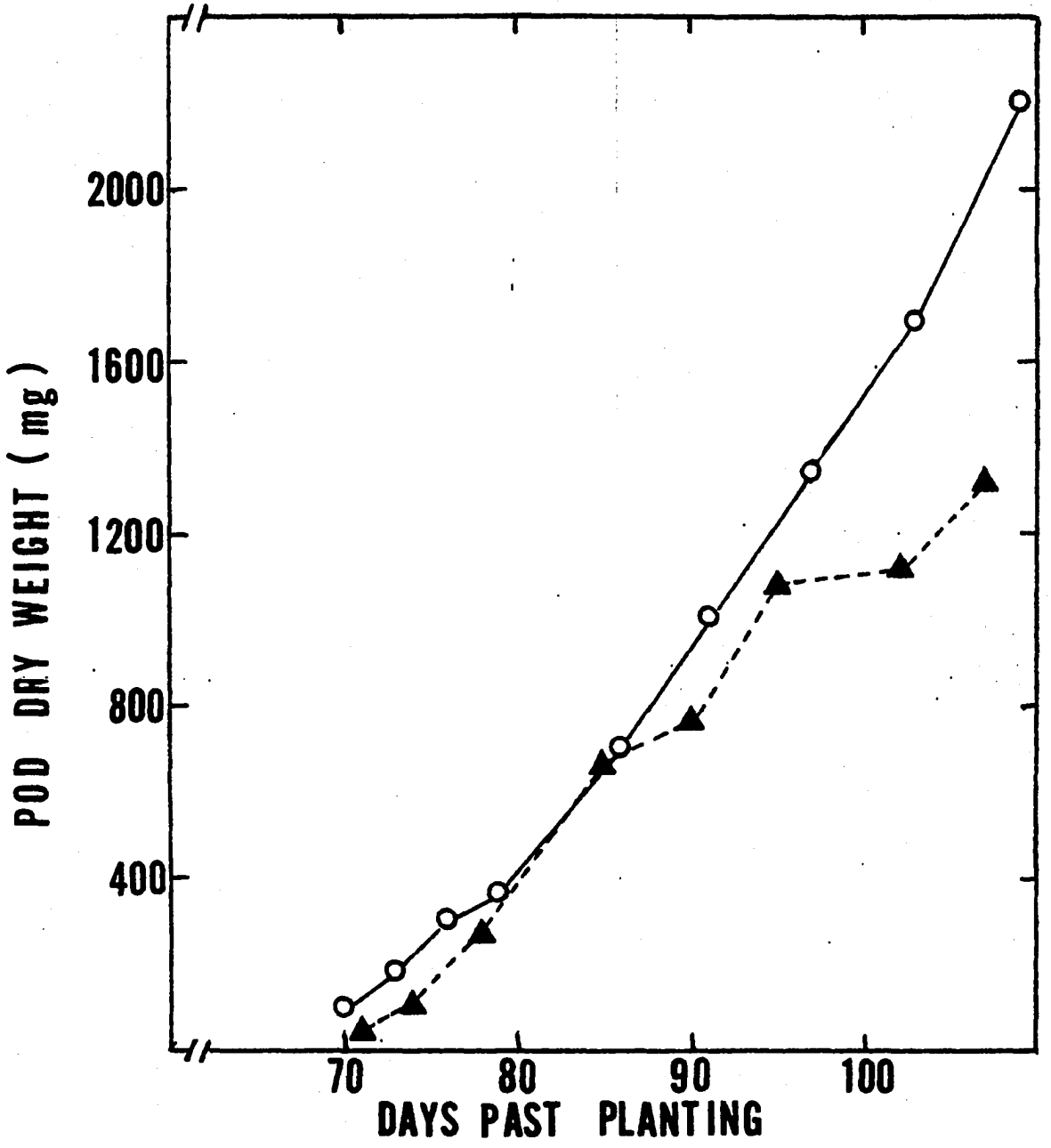


Figure 6. Seasonal trends of pod dry weight for nodes 12 (O) and 15 (▲)--1979

Physiological and Biochemical Parameters

Diffusive resistance to water

The diffusive resistance data are presented in Figure 7. Because (1) leaf diffusive resistance (r_1) is mathematically determined from abaxial (rab) and adaxial (rad) resistance and (2) rab and rad are highly correlated (r=0.88 and 0.99 for nodes 12 and 15, respectively), only the results of r_1 are discussed.

Leaves at both nodes had similar seasonal r_1 means and similar quadratic trends.

The L.S.D. test can be inaccurate for determining significant differences between two adjacent days if large, sequentially alternating high to low day-to-day variability exists among the data. One method that can be used to calculate differences more accurately between adjacent days is a sequential orthogonal contrast. This contrast is constructed such that a particular value for a day is tested against the mean of the previous day(s). So, for example, day 70 is compared with day 66, day 73 is compared with the mean of days 66 and 70, day 76 is compared with the mean of days 66, 70 and 73, and so on. This method detected a significant increase in r_1 in node-12 leaves between day 104 and the preceding days, whereas, the L.S.D. did not detect a significant increase until after day 104. In node-15 leaves, both the L.S.D. and the sequential orthogonal contrast

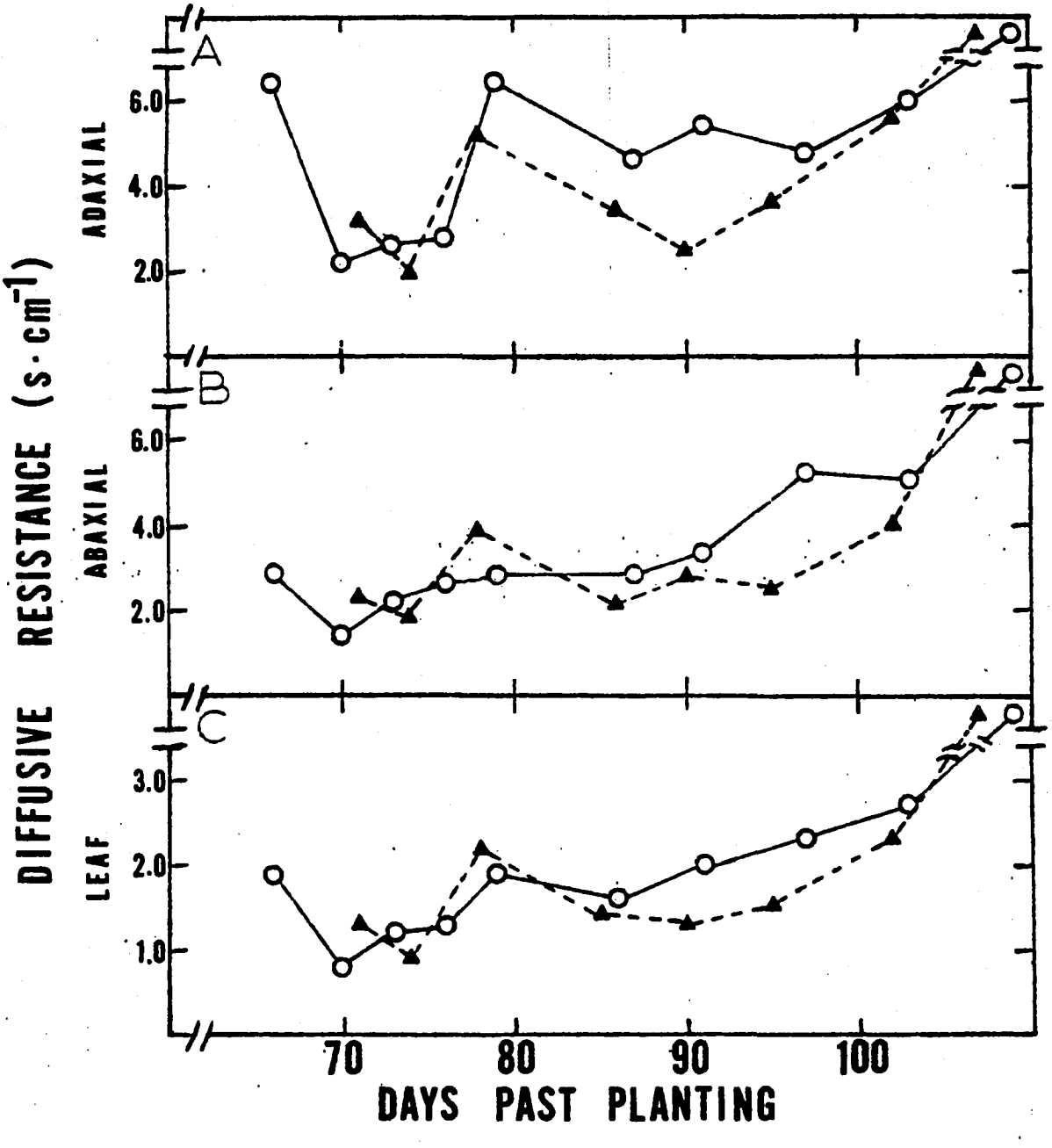


Figure 7. Seasonal trends of adaxial (A), abaxial (B) and total (C) leaf diffusive resistance to water for nodes 12 (O) and 15 (▲)--1979

detected an increase after day 102.

Carbon dioxide-exchange rate

Figure 8A depicts the seasonal behavior of carbon dioxide-exchange rate (CER). Leaves at node-15 had a greater seasonal mean CER than leaves at node-12, but when adjusted for differences in SLW, no significant differences existed.

The seasonal trend of CER for leaves at each node was quadratic, but the nature of the function or curve differed between node-12 and node-15 leaves. In leaves at node 12, a slow, steady increase in CER occurred until the maximum was reached on day 86. Then, CER began to decline slowly. Node-15 leaves, on the other hand, rapidly increased in CER until day 85. From day 85 to day 90, CER was steady, and then began to decline rapidly after day 90.

The onset of the decline in CER was detected, at the .06 level of probability, between days 86 and 91 in node-12 leaves. A sequential, orthogonal contrast between day 95 and the mean of days 85 and 90 detected a decrease, at the .04 level of probability, in node-15 leaves. In node-12 leaves, rate of the decline in CER from day 86 to day 109 was 3.8% per day, whereas in node-15 leaves, the rate of decline from day 90 to day 107 was 26% greater, or 4.8% per day.

Respiration

Leaf respiration was highly variable throughout the season (Figure 8B). In spite of this variability, leaves at

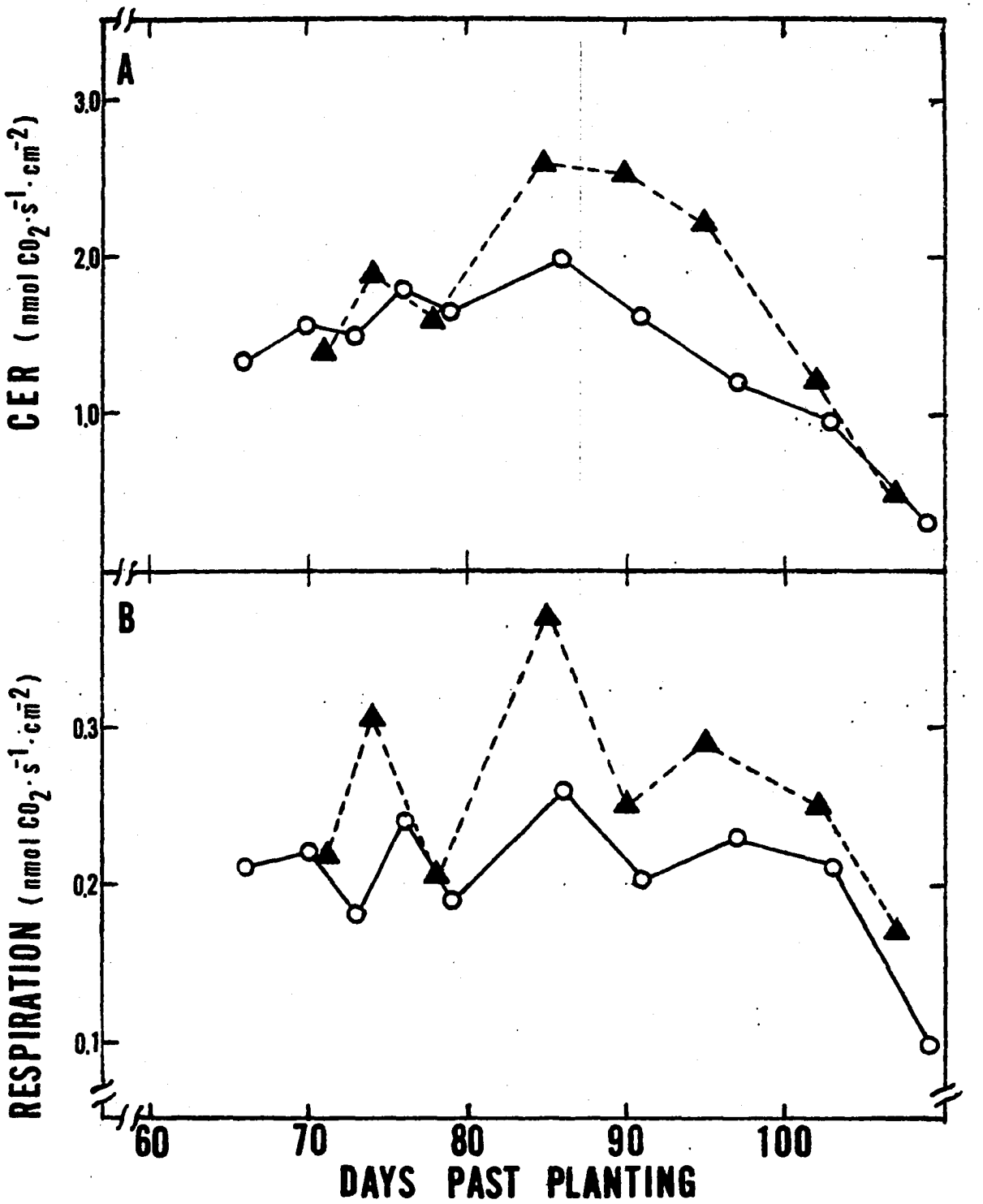


Figure 8. Seasonal trends of carbon dioxide-exchange rate (CER) (A) and respiration (B) for nodes 12 (O) and 15 (▲)--1979

node 15 had a greater seasonal mean than leaves at node 12. However, as with CER, when respiration was calculated on a leaf dry weight basis, no difference existed between the leaves at the two nodes.

Leaves from both nodes had a similar quadratic relationship over the season. Because of the high day-to-day variability in respiration, a series of sequential orthogonal contrasts was performed to determine when respiration began to deline. It did not decline significantly until after day 104 in node-12 leaves and after day 102 in node-15 leaves.

Chlorophyll

Seasonal changes in chlorophyll a content, chlorophyll b content, total chlorophyll content (CHL) and chlorophyll a:b ratio are shown in Figures 9 and 10. The correlation between chlorophyll a and chlorophyll b was very high for leaves at each node (r=0.99 for node-12 leaves and 0.98 for node-15 leaves) and, thus, results will be discussed in detail only for CHL (Figure 10B), which is the sum of chlorophyll a plus chlorophyll b.

CHL increased 43% from day 66 to day 76 in node-12 leaves, whereas a 31% increase from day 71 to day 78 was observed in node-15 leaves. After the rapid increase, CHL in both leaves was stable until about day 90, after which it began to decline. A slight decline in CHL was perceptible, at the .054 level of probability (L.S.D. and sequential

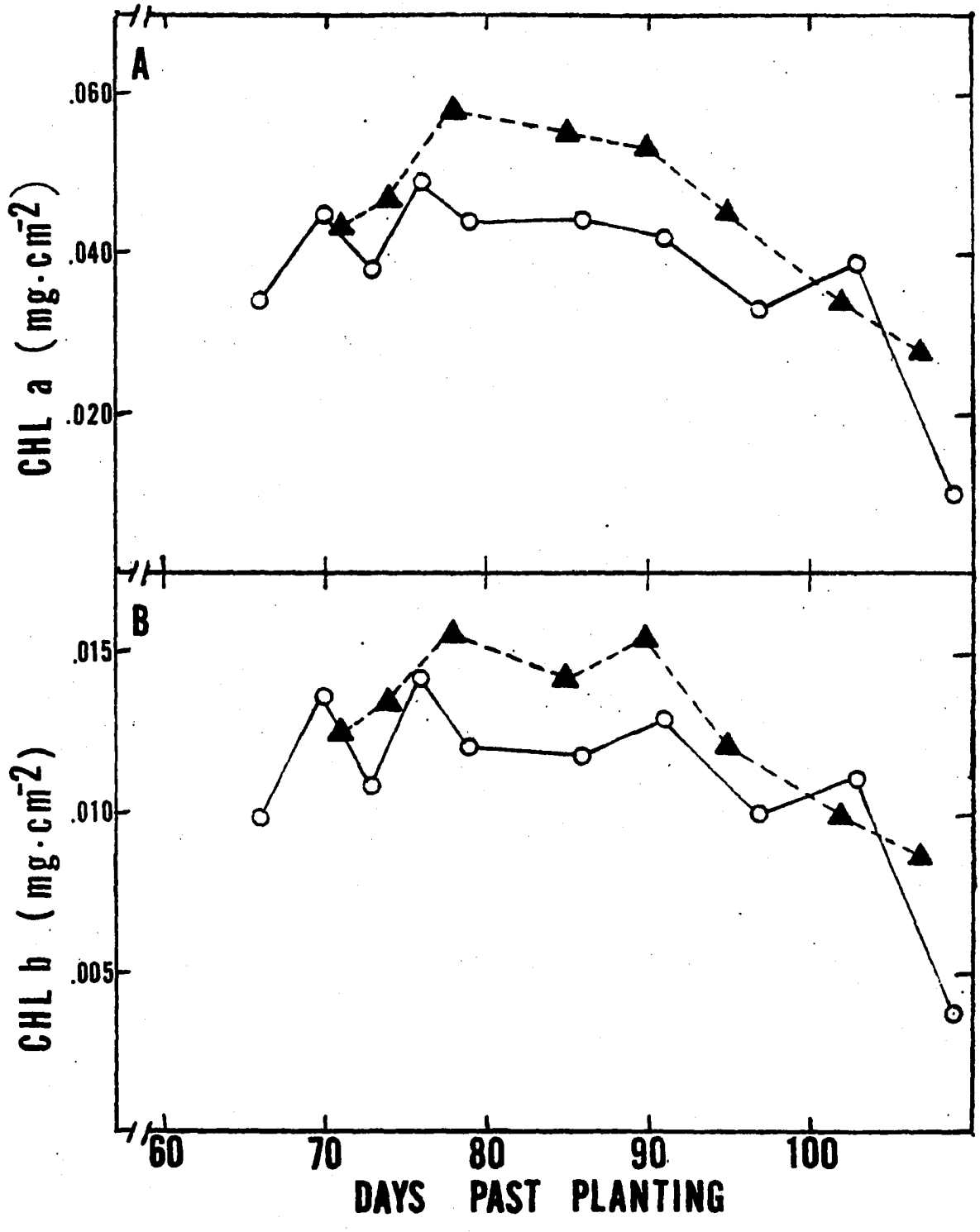


Figure 9. Seasonal trends of chlorophyll a (CHL a) (A) and chlorophyll b (CHL b) (B) for nodes 12 (○) and 15 (▲)--1979

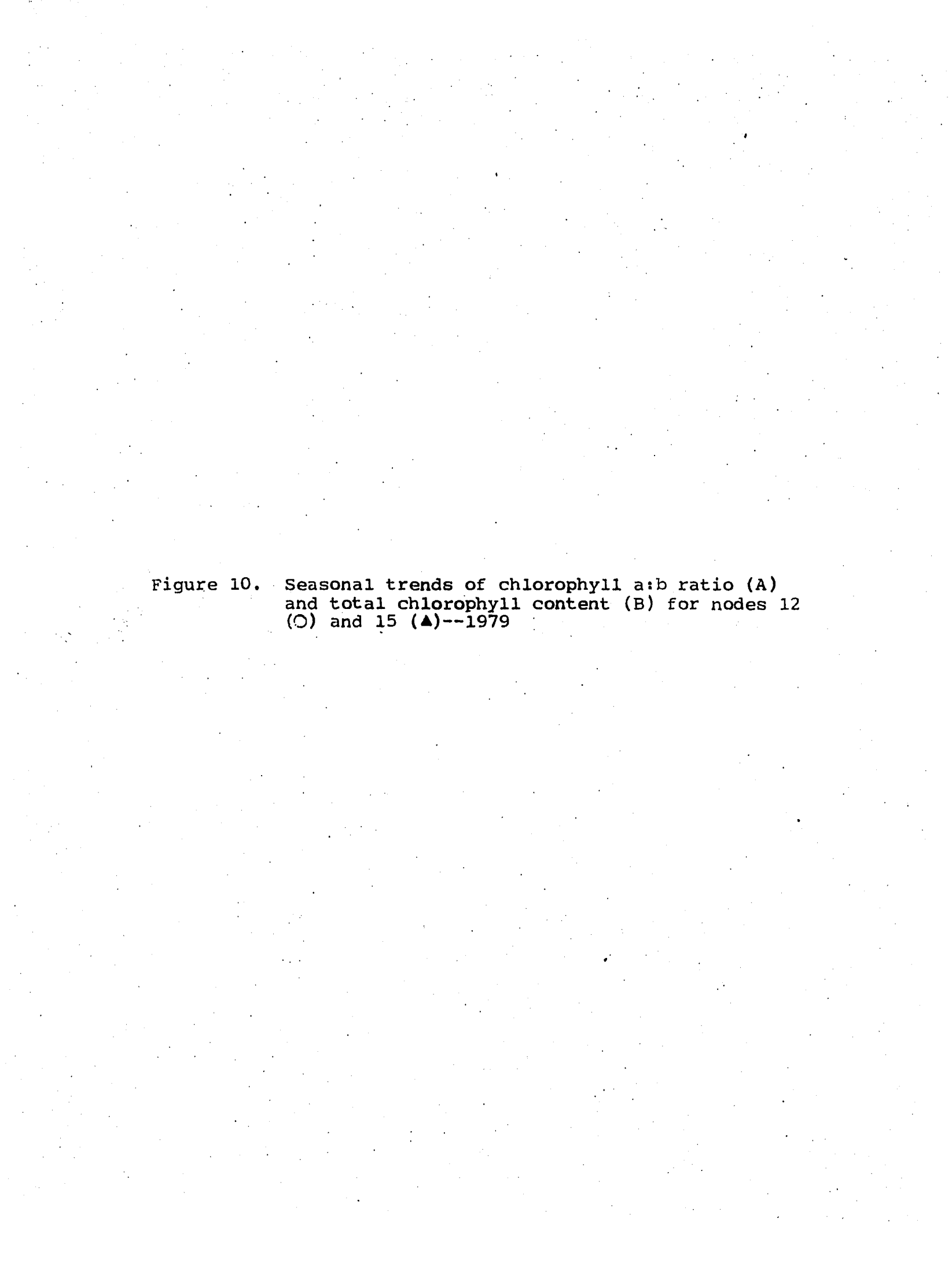
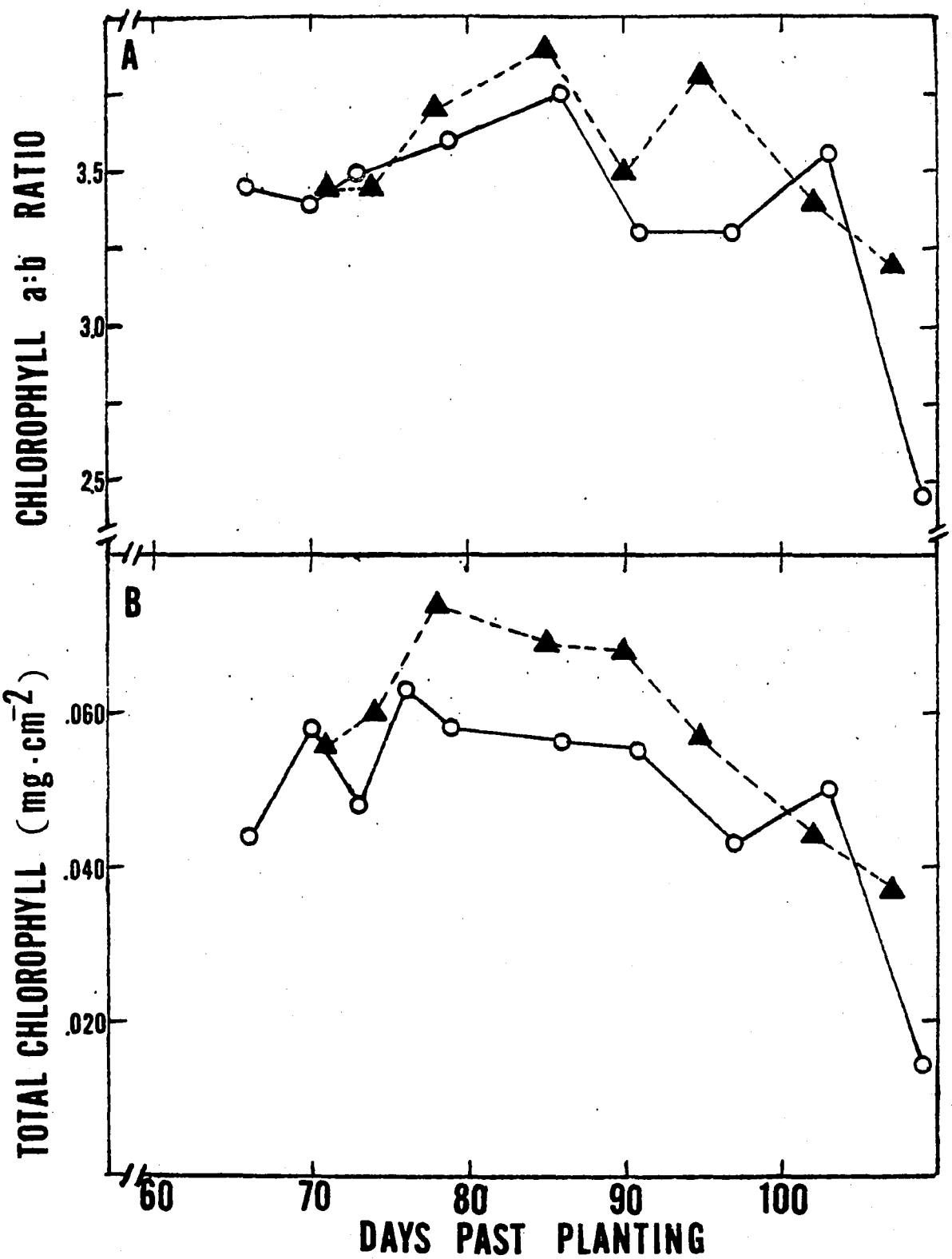


Figure 10. Seasonal trends of chlorophyll a:b ratio (A) and total chlorophyll content (B) for nodes 12 (○) and 15 (▲)--1979


A
B
CHLOROPHYLL a:b RATIO
3.5
3.0
2.5
TOTAL CHLOROPHYLL (mg·cm⁻²)
.060
.040
.020
60
70
80
90
100
DAYS PAST PLANTING

orthogonal contrast), between days 76 and 79 in node-12 leaves, but a highly significant decline did not occur until after day 91. CHL in node-15 leaves demonstrated a highly significant decline after day 90, as detected by a sequential orthogonal contrast.

The overall seasonal pattern of CHL was similar in leaves from both nodes, even though leaves from node-15 had a larger seasonal mean CHL than did node-12 leaves.

Figure 10A depicts the ratio of chlorophyll a to chlorophyll b over the season. Because the correlation between chlorophyll a and chlorophyll b is very high, one would expect a constant ratio between the two. According to a series of sequential orthogonal contrasts, no change in the ratio was evident at either node until the last respective sampling date. The lack of a differential degradation of chlorophylls agrees with the work of Patterson and Brun (1980), but not with Woolhouse (1974) or Sestak (1977).

Protein

The seasonal changes in crude protein content are shown in Figure 11A. On a leaf area basis, protein in leaves at node 15 was greater than in node-12 leaves, but when expressed on a leaf dry weight basis, protein was similar in leaves from both nodes.

Protein content in the leaves at node 15 increased 55% during the first three sampling dates, then remained constant

before beginning to decline after day 102 as determined by L.S.D.s. There was a rapid, significant increase in crude protein content in the leaves at node 12 between days 66 and 70, after which no discernibly significant changes occurred until day 109 when protein content had declined 50% from day 104.

There was no difference in the seasonal trend between the leaves at both nodes.

Ribulose 1,5-bisphosphate carboxylase

Ribulose 1,5-bisphosphate carboxylase (RuBPCase) activity, expressed on a leaf area basis, was parabolic over the season for leaves at each node (Figure 11B). The leaves from each node differed in seasonal mean RuBPCase activities, node-15 leaves having higher activity, and in the quadratic nature of the seasonal trend. Leaves at node 12 increased slowly from 1.93 nmol $CO_2 \cdot s^{-1} \cdot cm^{-2}$ on day 66 to a maximum of 5.37 nmol $CO_2 \cdot s^{-1} \cdot cm^{-2}$ on day 76. Thereafter, RuBPCase activity declined linearly, at a rate of 2.7% per day, to 0.51 nmol $CO_2 \cdot s^{-1} \cdot cm^{-2}$ on day 109. The increase in RuBPCase activity was less rapid in the leaves from node 15, increasing 90% from day 71 to day 85 (14 days) compared to the 178% increase during 10 days in the node-12 leaves. However, RuBPCase declined more rapidly in leaves from node 15 than node 12. From the maximum activity of 7.13 nmol $CO_2 \cdot s^{-1} \cdot cm^{-2}$ on day 85, RuBPCase activity in node-15 leaves declined to 1.54 nmol $CO_2 \cdot$

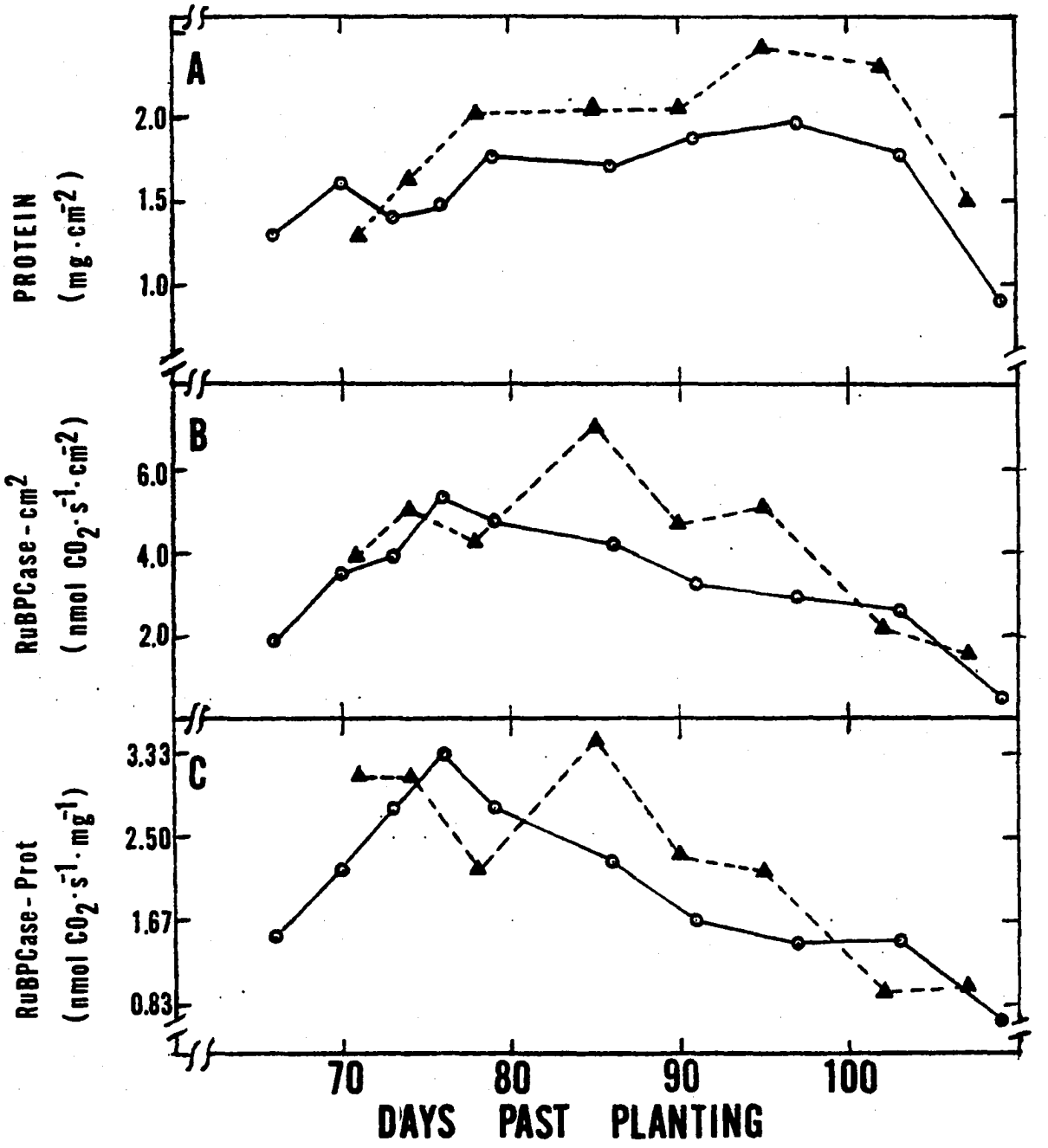


Figure 11. Seasonal trend of crude protein content (A), RuBPCase activity per leaf area (RuBPCase-cm^2) (B) and per mg crude protein (RuBPCase-Prot) (C) for nodes 12 (○) and 15 (▲)--1979

$s^{-1} \cdot cm^{-2}$ on day 107, or approximately a 3.6% decline per day.

Figure 11B shows clearly RuBPCase activity beginning to decline after day 76 in node-12 leaves and after day 86 in node-15 leaves. In leaves at node 15, the difference between RuBPCase activity on days 85 and 90 is significant as determined by a L.S.D. test. But the difference in activity between days 76 and 79 is not significant for the leaves at node 12. The first significant decline occurs on day 86 when compared, using an orthogonal contrast, to the mean of days 76 and 79.

The changes in RuBPCase activity can be attributed to changes in the amount of enzyme, the kinetics of the enzyme, or both. Expressing the activity of the enzyme on a protein basis can account for changes in the amount of the enzymes, particularly if the enzyme is a major constituent of the soluble leaf protein, as is RuBPCase in soybean leaves (Wittenbach et al., 1980). Unfortunately, the protein assayed in this experiment was crude, rather than soluble protein, and thus may not accurately represent the amount of RuBPCase present. Nevertheless, RuBPCase activity per unit protein (RuBPCase-Prot) data are shown in Figure 11C.

The seasonal trend in RuBPCase on a protein basis was well correlated with RuBPCase activity on a leaf area basis in leaves from both nodes; the correlation coefficient, r, was highly significant for each node: node 12 = 0.95, node

15 = 0.84. Since protein content was stable over most of the season, it seems that the changes in RuBPCase activity were attributable to changes in enzyme kinetics rather than protein amount.

Associations with CER

The two objectives of this experiment sought to determine which parameter(s) was best associated with decline in photosynthetic rate and to learn whether CER in leaves growing during the same plant developmental stage were influenced by similar factors. There are three methods available to examine the relationship between CER and other parameters. These methods are: (1) correlation, (2) regression, and (3) day-to-day analysis. According to Steel and Torrie (1960), "correlation is a measure of the degree to which variables vary together or a measure of the intensity of association" and that "where variables are jointly affected because of external influence, correlation may offer the most logical approach to an analysis of the data." Thus, if two variables are commonly affected by a third variable,then high correlations will result between the first two variables. Regression, on the other hand, deals "primarily with the means of one variable and how their location is influenced by another variable" (Steel and Torrie, 1960). Regression analysis is more a cause and effect technique, whereas correlation is

more descriptive. The biggest disadvantage with correlation and regression analyses is that they are purely mathematical functions, which require careful scrutiny when used in biological research. The third method, day-to-day or trend inspection, is more subjective than correlation and regression. A certain response or trend is expected because of a priori knowledge. Deviations from that response are investigated with respect to environmental or other aberrant effects. Points of inflection as well as day-to-day variability are tested using simple statistics such as L.S.D.s or orthogonal contrasts, which require prior biological (in this experiment) knowledge and justification for the test. I will use all three methods to analyze the data. First, I will present the day-to-day or trend inspection data and then use correlation and regression to support the results.

Day-to-day analysis

The summary of the day-to-day analysis is shown in Table 1. The day of decline is the sample day before a significant decline was detected statistically. The percentage decline of a parameter is calculated from day 86 to day 109 for node-12 leaves, and from day 90 to day 107 in node-15 leaves; i.e., from the day CER began to decline. I used the time of the onset in the decline of CER as a comparative basis because: (1) according to my definition, it is the time when leaf senescence begins, (2) it provides a relative basis for

Table 1. Summary of seasonal trends of several metabolic parameters

Parameter[a]	Node 12		Node 15		Node x function interaction
	Day of decline	% decline	Day of decline	% decline	
CER	86	87	90	86	Yes
CHL	76,91	75	90	46	No
RuBPCase-cm^2	86	88	85	66	Yes
RuBPCase-Prot	76	77	85	55	No
r_l	104	258[b]	102	377[b]	No
Protein	104	49	102	29	No
Rn	104	61	102	29	No

[a]Abbreviations used: CER, carbon dioxide-exchange rate; CHL, chlorophyll content; RuBPCase-cm^2, RuBPCase activity per leaf area; RuBPCase-Prot, RuBPCase activity per mg protein; r_l, leaf diffusive resistance to water; Rn, respiration.

[b]Percentage increase.

comparing the coincident decline in a parameter with the decline in CER, and (3) it establishes a uniform, nonarbitrary point from which comparisons can be made.

The data in Table 1 show that CHL, RuBPCase-cm^2 and RuBPCase-Prot begin to decline at about the same time CER did in each leaf, except possibly for CHL in node-12 leaves. CHL in node-12 data has two values because of the uncertainty in determining on which day the decline began. In leaves at

both nodes, the percentage decline in RuBPCase-cm^2 was most similar to the percentage decline in CER, though in node-15 leaves it did not decline as much as CER. Moreover, CER and RuBPCase-cm^2 are the only parameters that have a node by function interaction, meaning that the leaves at each node displayed different seasonal trends in CER and in RuBPCase-cm^2. RuBPCase-Prot in node-15 leaves began to decline on the same day as CER, but the percentage decline was quantitatively less than CER. The percentage decline in RuBPCase-Prot in node-12 leaves is not much less than CER. However, the onset of the decline preceded CER by 10 days. The relationship between RuBPCase-cm^2 and RuBPCase-Prot is remarkedly parallel (Figures 11A and 11C), as attested by high correlation coefficients of 0.84 and 0.95 for leaves at nodes 12 and 15, respectively. Part of the reason for the good relationship between RuBPCase-cm^2 and RuBPCase-Prot is that the basis for RuBPCase-Prot, crude protein content, was constant over the season. Thus, the decline in CER seems to be associated more with a change in specific activity of RuBPCase than with amount.

CHL and CER began to decline on the same day in node-15 leaves, but the percentage decline of CER is nearly twice that of CHL. In node-12 leaves, the percentage decline of CHL was closer to the percentage decline in CER than in node-15 leaves. Because there was no node by function interaction, I tend to

believe that CHL in node-12 leaves truly began to decline after day 90 (see Figure 10B). Another reason for believing this is that the decline in CHL after day 90 was far more statistically significant than the decline between days 76 and 79. If this is true, then the decline in CHL actually occurred after the decline in CER in node-12 leaves.

The significant changes in r_1, protein and Rn come too late to be considered causative factors in CER decline.

Correlation analysis

The second method used to analyze the data is correlation analysis. Table 2 displays the coefficients resulting from the correlation of several parameters with CER. The table has been divided into four sections, according to the days included in the analysis. The all-days period includes all the sampled days--the entire season. The before-day-107 period includes all days except the last for each node--day 109 for node-12 plants, day 107 for node-15 plants. The reason for omitting the last sampling day is that on this day some leaves were yellow and about to abscise. By this time, senescence was so far advanced in some leaves that the discernment of causal physiological parameters on CER was hampered by the physical deterioration of the leaves. The other two periods, before-day-95 and after-day-85, represent periods before and after, but both including the time when CER was maximum. The purpose for dividing the season into sections was to determine

Table 2. Correlations based on means from each day for several parameters with CER[a] for several periods

Parameter	Period: All days	Before-day-107	Before-day-95	After-day-85
		Node 12		
Rn	0.81**	0.37	0.71	0.87
CHL	0.91**	0.67*	0.73	0.90
RuBPCase-cm^2	0.86**	0.70*	0.67	0.97**
RuBPCase-Prot	0.78**	0.64	0.42	0.97**
r_1	-0.89**	-0.64	0.00	-0.93*
n	10	9	7	5
		Node 15		
Rn	0.76*	0.63	0.65	0.77
CHL	0.80**	0.62	0.45	0.98*
RuBPCase-cm^2	0.90**	0.84**	0.75	0.94
RuBPCase-Prot	0.66*	0.48	0.11	0.92
r_1	-0.80**	-0.53	-0.25	-0.91
n	8	7	5	4

[a]Abbreviations as per Table 1.

*,**Indicate statistical significance at the 0.05 and 0.01 levels of probability, respectively.

if the association between a parameter and CER varied within the season.

When all of the days are included in the correlation, all parameters are significantly correlated with CER. However, by omitting days 107 and 109, only CHL and RuBPCase-cm^2 in node-12 plants, and RuBPCase-cm^2 in node-15 plants, remain significantly correlated. In node-12 plants, RuBPCase-Prot and r_1 are very near to being significantly correlated with CER. But, because there is no significant increase in r_1 until day 107 (Table 1), the correlation is based on data points which are not statistically different; therefore, any causal relationship between r_1 and CER is rather tenuous. That (1) the correlation between Rn and CER is low and not significant in the before-day-107 data, (2) Rn does not significantly change until very late in the season (Table 1), and (3) Rn decreases, rather than increases, indicate that Rn does not account for decreasing CER.

None of the parameters is significantly correlated with CER before day 95. In node-15 leaves, RuBPCase-cm^2 has the best correlation with CER ($r=0.75$), but partly because of the low sample size ($n=5$), it is not significant. Another reason for the nonsignificant results early in the season is that there may be a poor association between CER and the parameters during the anabolic phase of leaf development, because, although CER is functionally related to CHL and RuBPCase-cm^2,

an abundance of CHL and a potentially high RuBPCase activity suggest these may not be limiting to CER in young leaves.

During the senescence phase of leaf ontogeny, after day 85, a strong, significant association exists between CER and RuBPCase-cm^2, RuBPCase-Prot, CHL, and r_1. Although other correlations are high, one reason that they are not significant is the low number of observations in the after-day-85 data. It is difficult to draw conclusions from this senescence-phase data because many correlations are high and there is not much of a quantitative difference between the significant and nonsignificant correlations. During the senescence phase of leaf ontogeny, many of the parameters may decline, not necessarily through direct associations with each other, but because, particularly in the later stages of senescence, of the general physical deterioration of the leaf. Regression analysis in the following section will be used to define quantitatively the magnitude of these associations.

Regression analysis

One of the shortcomings in using correlation analysis is that it does not detect or measure the magnitude of the effect one variable exerts on another. The use of regression overcomes this shortcoming by estimating the relationship between two variables. If the regression coefficient or the slope of the regression line is not different from zero, then no causal

relationship can be inferred between two variables, regardless of the significance of their mutual correlation. Table 3 shows the results, based on before-day-107 and after-day-85 data, of regressing CER on several parameters. The parameters in Table 3 are those that have been shown by the day-to-day analysis to have a seasonal trend similar to CER.

CHL and RuBPCase-cm^2 in node-12 leaves had regression coefficients significantly different from zero in both the before-day-107 and the after-day-85 data. Additionally, during the senescence phase, after-day-85, RuBPCase-Prot in node-12 leaves had a regression coefficient that was different from zero. Thus, the data suggest that CHL and RuBPCase activity are definitely associated with CER throughout the season. The associations are statistically stronger during the senescence phase. That protein content remains constant (Figure 11A) and that RuBPCase-cm^2 and RuBPCase-Prot are highly correlated ($r=0.93$ and 0.99 for before-day-107 and after-day-85, respectively) imply that the decline in CER is caused by a decline in RuBPCase specific activity rather than amount.

Less statistically significant results are demonstrated in the node-15 data, partly because of the small sample size. According to the coefficient of determination, r^2, 71% of the variation in CER can be accounted for by RuBPCase-cm^2 in the before-day-107 data. All three parameters in the

Table 3. Regression coefficients from the regression of CER[a] on several parameters

Data set	n	CHL		RuBPCase-cm^2		RuBPCase-Prot	
Before-day-107							
Node 12	9	30.9±12.4[b]*	(0.47)[c]	0.19±0.07*	(0.50)	0.25±0.11	(0.41)
Node 15	7	32.9±18.8	(0.41)	0.30±0.09*	(0.71)	0.30±0.24	(0.23)
After-day-85							
Node 12	5	33.4±9.5*	(0.81)	0.47±0.07**	(0.94)	0.99±0.15**	(0.93)
Node 15	4	66.4±9.8*	(0.96)	0.50±0.13	(0.88)	1.19±0.37	(0.84)

[a]Abbreviations as per Table 1.

[b]Standard errors of the estimate.

[c]r^2.

*,**Indicate regression coefficient significantly different from zero at the 0.05 and 0.01 levels of probability, respectively.

senescence phase data have high r^2 values with CHL having the highest (0.96). The regression coefficients for RuBPCase-cm^2 and RuBPCase-Prot were statistically different from zero at less than the 0.10 but greater than the 0.05 level of probability. Because of their high r^2 values, RuBPCase-cm^2 and RuBPCase-Prot are important in describing the changes in CER. For the same reasons as I pointed out for the node-12 data, the node-15 data suggest that decline in CER is strongly influenced by the decline in CHL and RuBPCase specific activity.

The physiological implications and interactions among CHL, RuBPCase and CER will be discussed in a later section.

Relationship Between Nodes

The seasonal mean of the physiological and metabolic parameters differed between the leaves. The difference was attributable to the differences in SLW between the leaves at the nodes. Leaves at node 15 had greater CER, CHL, RuBP-Case-cm^2 and protein on a leaf area basis but not on a leaf dry weight basis.

Photosynthesis began to decline on nearly the same day in leaves at each node. The other parameters began to decline on similar days in leaves at both nodes. As shown in Table 1, only two parameters, RuBPCase-cm^2 and CER, had a significant node by function interaction; all the other parameters had

similar trends over the season for both nodes. The difference between node-12 and node-15 leaves in seasonal CER trend can be attributed to the steeper rate of incline and decline in CER. Although the rate of increase in RuBPCase-cm^2 is nearly the same in the leaves at nodes 12 and 15 (Figure 11B), the rate of decline in node-15 leaves is 50% greater than in node-12 leaves. Thus, the leaves at the two nodes differ somewhat with respect to the nature in which CER and RuBPCase-cm^2 behave over the season.

But there are no differences between leaves at the two nodes with regard to the strength of the relationship between CER and the other parameters. From Table 3 it can be seen that there are no significant differences between respective regression coefficients in the leaves at nodes 12 and 15. Thus, the relationship between a given parameter and CER is the same for leaves at both nodes. This occurs even though some, actually most, of the relationships are not significantly related to CER. This can be interpreted to mean that the same or similar causal phenomena are operative in both leaves but some are more important than others in ultimately effecting changes in CER.

PART II. COMPARISONS AMONG LEAVES OF DIFFERENT PLANT ONTOGENY

EXPERIMENTAL PROCEDURE

Plant Material and Culture

In 1980, the plant material was grown in the same two wooden bins as was the 1979 plant material. The soil mixture used in 1979 was removed from the bins and a new mixture containing 2 parts soil:1 part peat:1 part sand was used to refill the bins. Fertilizer, prepared by dissolving 120 g KH_2PO_4 and 80 g K_2SO_4 in 2 liters of water, was applied to each bin in trenches approximately 11 cm from the inside bin wall and approximately 18 cm below the soil surface.

'Amsoy-71' soybean seeds, derived from the 1979 plants, were planted on 27 May 1980 at the rate of 12 seeds per 30 cm in a single row along the center of each bin. On 7 June 1980, I thinned to 6 plants per 30 cm. Care was taken during the thinning to keep plants which were uniform in size and development. On 18 June, a severe hailstorm damaged many of the plants, so I replanted seeds on 19 June. The second planting was thinned on 30 June, when the first trifoliolate leaves began to emerge.

Weeding, irrigation and insect control were the same as in 1979, except that Cygon 2-e (dimethoate 0,0-dimethyl S-[(methyl-carbamoyl) methyl] phosphoro dithioate) was used as a miticide in the beginning of the season. Later in the season, Plictran was applied instead of Cygon 2-e.

Sampling Protocol and Procedures

Plant selection and sampling

One hundred plants in each bin were numbered and tagged. Ten unnumbered plants at both ends of each bin served as border plants. Leaves at the third, eighth and thirteenth nodes (first trifoliate leaf = node 1) were to be sampled. As these leaves emerged, I recorded the data for each plant. To minimize variation in leaf age, I sampled only node-3 leaves that emerged on day 18 (18 days after planting), only node-8 leaves that emerged on days 34 and 35 and only node-13 leaves that emerged on days 49 through 52. Sampling order was at random, and only healthy, normal plants were sampled.

There were 36 sampling days over the season: 10 for node-3 leaves, 15 for node-8 leaves and 11 for node-13 leaves. The sampling periods were from day 25 to day 54 for node-3 leaves, from day 39 to day 92 for node-8 leaves and from day 60 to day 96 for node-13 leaves. Thus, samples were taken, on the average, about every 3 days at each node. The leaves at nodes 3 and 13 developed during the vegetative and seed-filling periods, respectively, whereas leaves at node-8 developed during an overlapping period.

Leaves from a specific node from each of four plants were sampled on a given day, that is, leaves from different nodes were never sampled on the same day. The leaves from node 3 and node 8 were never sampled from the same plant. Thirteen

plants had both their third and thirteenth leaves sampled; the thirteenth leaf was sampled at least 5 weeks after the third leaf was sampled on the same plant. No effects of previous sampling were expected. Two plants had both their leaves at nodes 8 and 13 sampled, but on different days. This was because of an oversight. So, a total of 143 samples were taken from 128 plants on 36 days. The remaining 72 tagged plants were not used because of abnormal development or growth, or missing leaves.

Sampling started on 15 July, 25 days after planting, and continued until 24 September, 96 days after planting. Sampling began at a given node (3, 8 and 13) when the leaves at that node were large enough to cover the window area of the gas-exchange leaf chamber. Figure 12 shows the general protocol used on each day. On the day before sampling, the leaf at the node to be sampled was tagged on a randomly selected, acceptable (proper day of leaf emergence, healthy) plant. On the sampling day, after the preparatory work was completed, diffusive resistance and CER (carbon dioxide-exchange rate) were measured, in situ, on the terminal leaflet of the tagged leaf. Following the in situ measurements, two leaflets and the pods at the node, if present, were brought into the laboratory to be analyzed for biochemical and physiological parameters as described later.

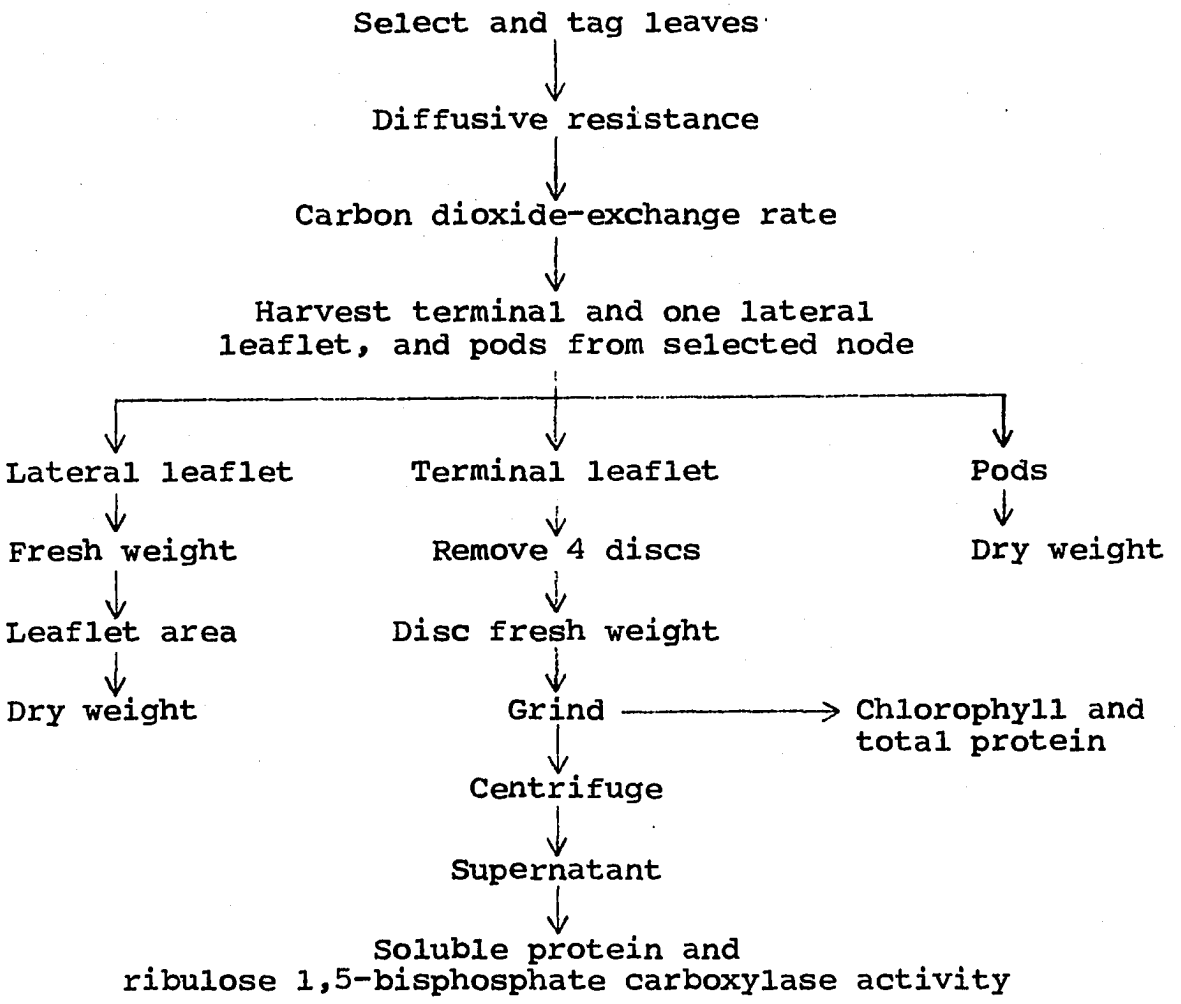


Figure 12. Sampling protocol--1980

Plant Measurements

Diffusive resistance

Diffusive resistance was measured in the same manner as in Part I.

Gas exchange

The gas exchange system and the procedure for measuring CER were essentially the same as in Part I, except that the infrared gas analyzer was calibrated with 327 $\mu l \cdot l^{-1}$ and 286 $\mu l \cdot l^{-1}$ CO_2 in N_2 primary grade standard gases (Matheson, Joliet, Illinois).

Plant and leaflet data

As was done in Part I, the leaf number from the apex was recorded during the gas exchange measurements. Because an additional leaflet was harvested in this experiment, a slightly different procedure was employed following the conclusion of the CER measurements. After the CER measurements were completed for the four plants, the left lateral leaflet (as referenced from the plant stem) of each sampled leaf was removed, placed in a moist plastic bag, and brought into the laboratory. The fresh weight of the leaflet was measured, after which the area of the leaflet was recorded. The lateral leaflet was then placed into a test tube, dried at 70 C for at least 24 h and then weighed. Specific leaf fresh weight (SFW) and specific leaf dry weight (SLW) were

calculated by dividing the fresh and dry leaflet weights, respectively, by leaflet area.

Pods, if present at the leaf node, were removed at the time the lateral leaflet was harvested, placed into a labelled test tube and later dried at 70 C for at least 24 h.

Biochemical and Analytical Assays

Tissue preparation

Each terminal leaflet was harvested, processed and the extract assayed before the next leaflet was removed from the plant. This procedure was performed to keep the leaf material as fresh as possible.

After a terminal leaflet was harvested, it was brought into the laboratory where four 3.63 cm^2 leaf discs were removed from the interveinal portion of the leaflet by using a no. 15 cork borer. The fresh weight of the leaf discs was recorded before they were sliced into small pieces. The sliced leaf material was transferred into a cold 18 x 150 mm culture tube to which was added 5 ml of 4 C grinding buffer (pH 7.8) containing 25 mM Tris, 25 mM NaH_2PO_4, 10 mM $MgCl_2$, 0.1 mM EDTA, 20 mM $NaHCO_3$ (fresh), 10 mM 2-mercaptoethanol (fresh) and 2% PVPP. The leaf material was ground for approximately 2 min using a Polytron (Brinkman, Westbury, N.Y.) at about 3/4 full speed. After aliquots for chlorophyll and total protein determinations were removed from the resulting

extract, it was centrifuged at 5000 x g for 10 min at 4 C. The supernatant was assayed for soluble protein content and ribulose 1,5-bisphosphate carboxylase (RuBPCase) (E.C.4.1.1.39) activity.

Chlorophyll

Two 250 µl aliquots of crude extract, were pipetted into separate centrifuge tubes, and 3 ml of acetone were then added. The tubes were vortexed, stoppered and stored at 5 C in a dark refrigerator. The chlorophyll content was assayed the next day in the same manner as in Part I.

Protein

Two estimates of protein were determined, total (TP) and soluble (SP), using two techniques, a modified Lowry (Miller, 1959) and a modified Bradford (Bio-Rad Laboratories, Richmond, California).

The Bradford method was used because of a report that the Lowry assay inaccurately estimates protein content in senescing soybean leaves (Wittenbach et al., 1980). Additionally, the Bradford method is exceptionally easy and rapid to use.

For each technique used, duplicate determinations were performed; therefore, four aliquots were assayed each for TP and for SP. To estimate total protein, a 100 µl aliquot was removed from the crude, uncentrifuged extract and pipetted

into a test tube, after which it was diluted with 250 µl of water. The same procedure was followed for determination of soluble protein, except the aliquot was removed from the centrifuged supernatant. The test tubes (32 per day) were stoppered and stored at 5 C in a dark refrigerator.

The samples to be assayed by the Lowry method were precipitated with Na decholate and trichloroacetic acid according to the procedure of Bensodoun and Weinstein (1976). The precipitate was solubilized in 1.0 ml of 0.3 N NaOH before being assayed. The samples to be assayed by the Bradford method were taken directly from the diluted aliquots. All the protein samples were at room temperature before being assayed. The Bradford reagent and bovine serum globulin, which was used as a protein standard for both techniques, were purchased from Bio-Rad (Richmond, California).

After day 78, newly prepared Lowry reagents did not give the same results on the standard protein as did the previous reagents. Repeated preparations of the Lowry reagents failed to resolve the problem. Fortunately, the Bradford method continued to give consistent standard curves throughout the season. A correlation between the Lowry and the Bradford methods, based on individual samples taken before day 76, indicated a highly significant association. The correlation coefficient between the two methods was 0.87 for TP and 0.96 for SP. Therefore, I decided to discontinue the Lowry method after day 76.

RuBPCase activity

Ribulose 1,5-bisphosphate carboxylase activity was assayed in the same manner as in Part I, except for the following minor differences:

1. The scintillation vials were not flushed with N_2 before use.
2. A background assay (minus ribulose 1,5-bisphosphate) was performed, in addition to the duplicate assays, for each extract.
3. The enzyme mixture (extract, $H^{14}CO_3^-$, Tris buffer, $MgCl_2$) was incubated for 12 min in a shaking water bath at 25 C.
4. Ribulose 1,5-bisphosphate was dissolved in 20 mM Tris (pH 8.2) rather than in deionized, distilled water.
5. After day 81, Eastman Ready-to-Use III (Eastman Kodak Comp., Rochester, N.Y.) liquid scintillation cocktail replaced Handifluor.

RESULTS AND DISCUSSION

The statistical analyses presented in Appendix Table A5 contain the results used to compare the seasonal means. The means for each sampling day and the respective L.S.D. for each parameter are in Appendix Tables A6, A7 and A8.

Data common to the entire experiment are as follows. The leaves at nodes 3, 8 and 13 emerged, on the average, 18, 35 and 51 days after planting. Beginning bloom, the day when at least one flower was visible on each plant, occurred approximately 34 days after planting (24 July 1980).

Physical Parameters

Leaf number

The seasonal change in the position of a node relative to the apex is shown in Figure 13A. Throughout the period when leaves at node 3 were being sampled, new leaves at the apex were emerging at the rate of 3½ per day. The same rate of apical leaf emergence occurred during the node-8 sampling period until about day 65, after which no new apical leaves appeared and node-8 leaves remained about nine nodes from the apex. The node-13 data indicate that after day 63, the position of leaves at that node did not change statistically. Thus, leaves at node 3 were sampled during plant elongation, leaves at node 13 were sampled after elongation ceased, and leaves at node 8 overlapped both periods.

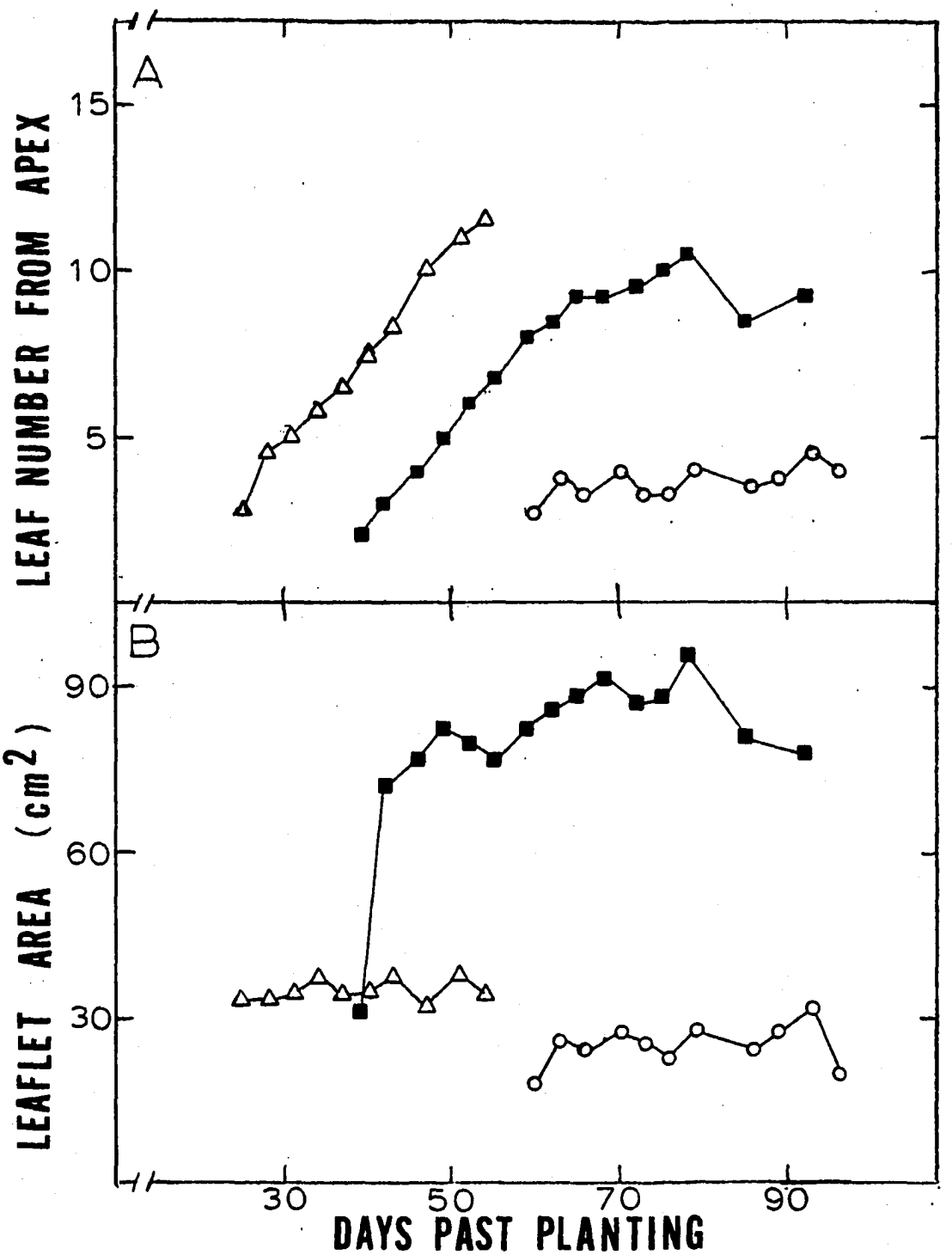


Figure 13. Seasonal trends of leaf number (A) and leaflet area (B) at nodes 3 (Δ), 8 (■) and 13 (o)--1980

Leaflet area

As is illustrated in Figure 13B, leaflet area was constant over much of the sampling period for each node. Using sequential, orthogonal contrasts, no statistically significant changes in leaflet area could be detected after day 25, day 46 and day 63 in leaves at nodes 3, 8 and 13, respectively. There was a difference in mean area among all three leaflets (Table A5, Appendix).

Pod dry weight

There was no substantial accumulation of pod dry weight at node 3. A rapid increase in pod weight had begun, on day 52, at nodes 8 and 13 (Figure 14A), and from day 55 onward, pods at both nodes had a similar growth rate of about 50 $mg \cdot day^{-1}$.

Comparison of Figures 13B and 14A indicates that node-3 leaves developed and began to senesce during vegetative plant ontogeny, node-13 leaves developed and lived during the reproduction period, and node-8 leaves overlapped both periods.

Specific leaf dry weight

The seasonal trends for specific leaf dry weight (SLW), the mass of dry leaf tissue per unit leaf area, are depicted in Figure 14B. Significant differences existed among the seasonal means for leaves at each node (Table A5, Appendix).

In leaves at node 3, SLW increased from day 25 to day 28

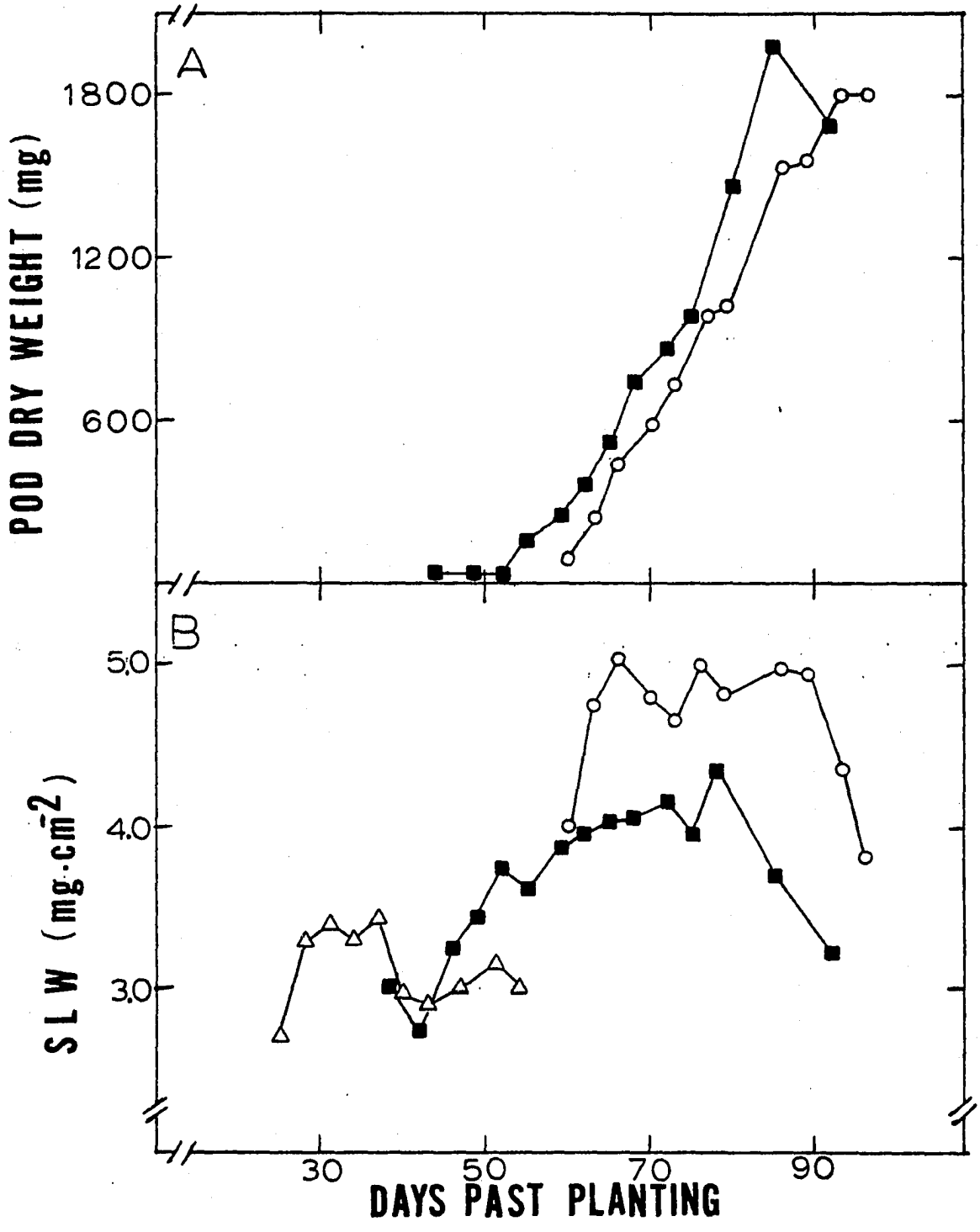


Figure 14. Seasonal trends of specific leaf dry weight (SLW) (A) and leaflet area (B) at nodes 3 (Δ), 8 (■) and 13 (o)--1980

before declining after day 37 to an essentially constant level for the remainder of the sampling period. A slow, steady, incremental increase was observed in node-8 leaves until day 78, after which a significant decrease occurred. A seasonal pattern different from either of the other two nodes was observed in leaves at node 13 where SLW increased rapidly between days 60 and 66, and remained essentially constant until day 89, after which it declined rapidly.

Physiological Parameters

Carbon dioxide-exchange rate

The seasonal patterns of carbon dioxide-exchange rate (CER) are illustrated in Figure 15A.

CER in node-3 leaves seems to have increased (though the change was not statistically significant) to a maximum on day 31 before linearly declining at the rate of 2.7% per day over the following 23 days.

The seasonal trend of CER in leaves at node 8 is more complicated. After CER rapidly increased to a maximum on day 52, there seemed to be three stages of decline: stage 1, from day 52 to day 59, during which CER declined at a rate of 3.7% per day; stage 2, from day 59 to day 78, during which CER declined about 0.5% per day; and stage 3, after day 78, during which CER declined at about 5.6% per day. Differences in CER between days 52 and 55, and between days 78 and 85 are

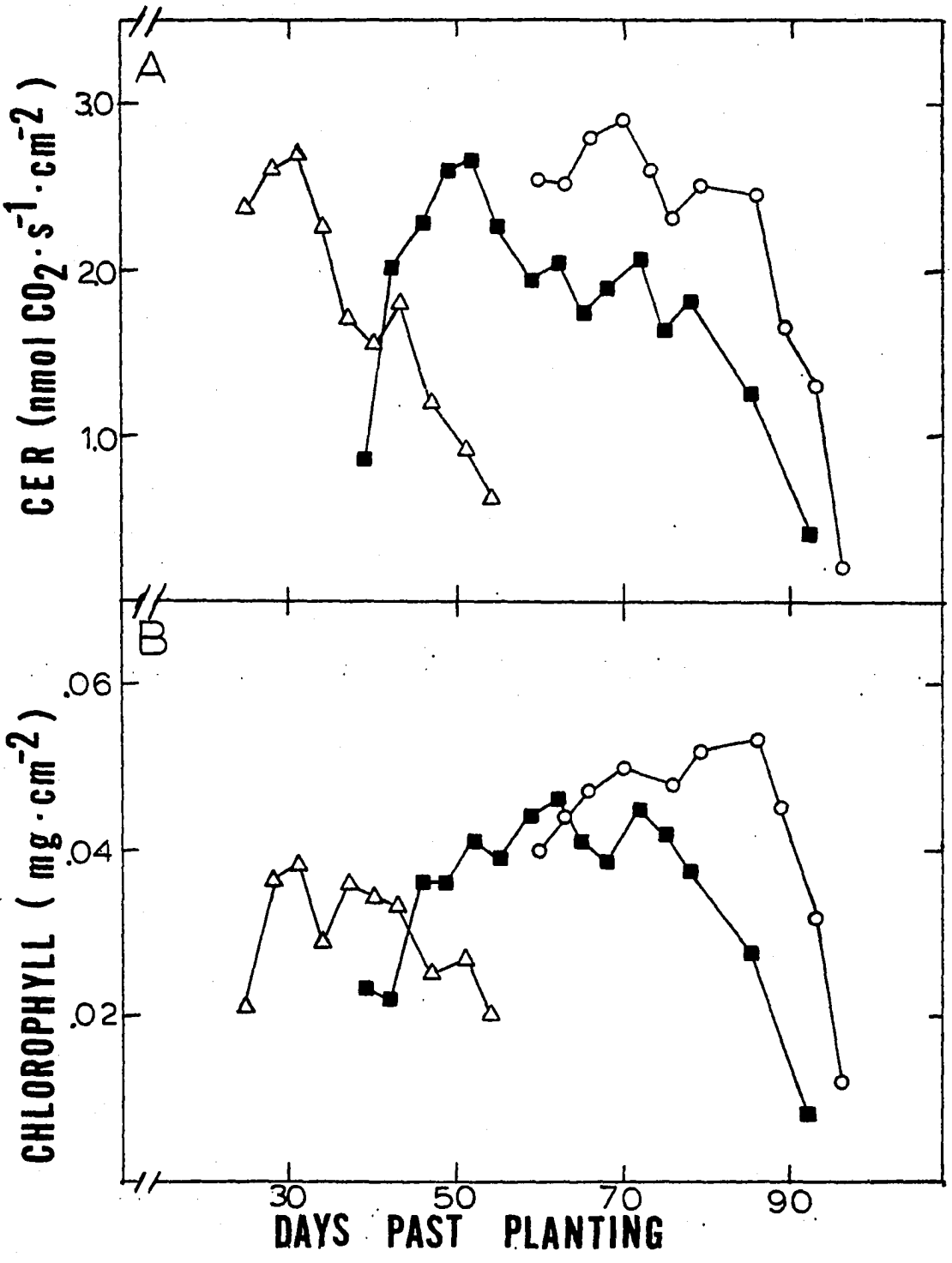


Figure 15. Seasonal trends of carbon dioxide-exchange rate (CER) (A) and chlorophyll (B) at nodes 3 (Δ), 8 (■) and 13 (o)--1980

significant at less than the 8% level of probability, using an L.S.D. test.

By the time the first measurement was taken on node-13 leaves, on day 60, CER was near its maximum, which occurred on day 70. The day-to-day variation in CER from day 60 to day 86 was not significant. But, following day 86, CER declined very rapidly at the rate of 9.2% per day.

Chlorophyll

Chlorophyll a and chlorophyll b content in the same leaflet were highly correlated (r=0.95, 0.97 and 0.98, for leaves at nodes 3, 8 and 13, respectively). Therefore, only total chlorophyll content (CHL), the sum of chlorophyll a plus chlorophyll b, will be discussed. Figure 15B shows the seasonal trend for chlorophyll in leaves at each node.

CHL in node-3 leaves reached a maximum on day 31, the same day CER in those leaves reached its maximum, then began to decline slowly. The datum for day 34 is apparently aberrant, inasmuch as CHL on day 31 does not differ from any of the three sampling days after day 34. Nevertheless, CHL declined a total of 48% from day 31 to day 54.

After increasing rapidly, CHL in node-8 leaves maintained a relatively constant level from day 52 to day 75, according to a sequential,orthogonal contrast which detected no statistically significant differences over this period, but did show that CHL had declined by day 78.

Compared to leaves at the other two nodes, node-13 leaves had a slower, more gradual increase in CHL. According to a sequential orthogonal contrast, no differences existed until day 86 when CHL had already begun to decline rapidly and linearly at the rate of 7.8% per day.

Leaf diffusive resistance to water

The seasonal trends of leaf diffusive resistance to water (r_l) can be seen in Figure 16A.

The r_l data for node-3 leaves appear to have a linear trend, but upon closer inspection, two groups of data seem to be present, separated by the only statistically significant day-to-day change, which occurred between days 37 and 40. No days before day 40 differed and no days after day 37 differed among themselves.

In node-8 leaves, a rapid decrease between the first two sampling days, followed by a slower decrease over the next three sampling days is observed for r_l. Regardless of these and other apparent trends, no statistically significant day-to-day changes from day 42 to day 85 are detected by a L.S.D. or by a sequential, orthogonal contrast. The only significant increase occurs between days 85 and 92.

Though r_l appears to be declining from day 60 to day 89 in node-13 leaves, no significant day-to-day differences exist until after day 93.

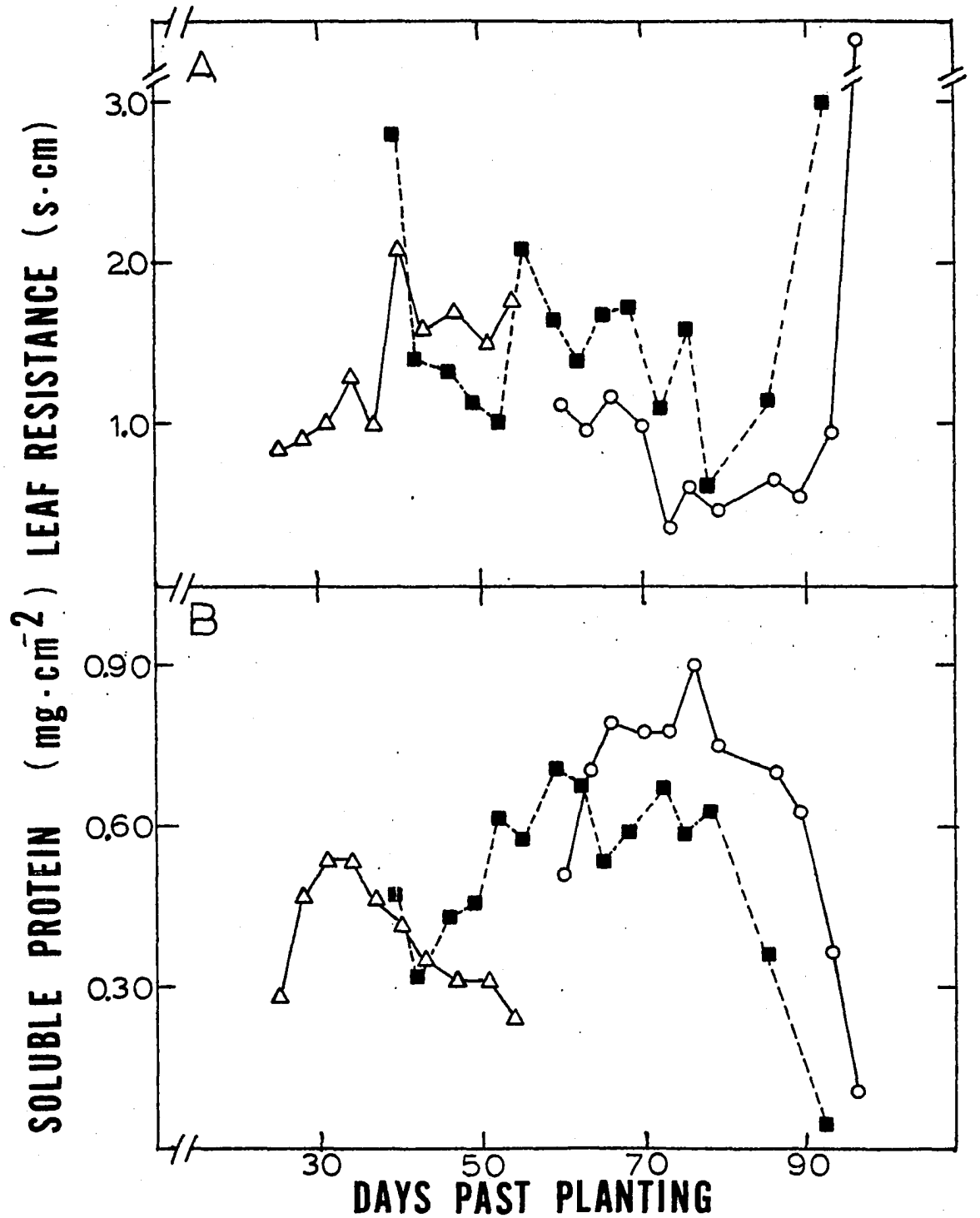


Figure 16. Seasonal trends of leaf resistance to water (A) and soluble protein (B) at nodes 3 (Δ), 8 (■) and 13 (o)--1980

Protein

Because soluble protein was highly correlated with crude protein (r=0.98, 0.99 and 0.99 for nodes 3, 8 and 13, respectively), only the soluble protein data will be discussed. The seasonal trends for soluble protein are illustrated in Figure 16B.

Protein in node-3 leaves produced a very smooth seasonal trend, increasing 80% to a maximum on days 31 and 34, followed by a steady, gradual decline at the daily rate of 2.8%.

There was more variability in the node 8 protein data than at node 3. Protein declined between the first two sampling dates, which could be partly accounted for by the decline in SLW over the same period. Apparently, leaf expansion was occurring at a rate greater than protein synthesis and dry matter accumulation. After increasing, protein content remained constant from day 52 to day 78, as verified by a sequential, orthogonal contrast. The decline in protein after day 78 occurred at a rate of about 6.6% per day.

In node-13 leaves, protein increased 54% from day 60 to day 66. The peak on day 76 is not significantly different from the days on either side of it. Although protein seems to decline irreversibly from day 76, no significant changes are detected until after day 89. The rate of protein degradation from day 86 onward is 8.7% per day.

Ribulose 1,5-bisphosphate carboxylase

Seasonal trends in ribulose 1,5-bisphosphate carboxylase (RuBPCase) (E.C.4.1.1.39) activity are shown in Figure 17. RuBPCase activity on a leaf area basis (RuBPCase-cm^2) is depicted in Figure 17A, whereas RuBPCase specific activity (RuBPCase-Prot), that is, RuBPCase activity expressed on a soluble protein basis, is shown in Figure 17B.

After an increase of 130% over 6 days, RuBPCase-cm^2 reached a maximum on day 31 in node-3 leaves, then declined rapidly to day 34. Thereafter, the activity declined slowly to its minimum on day 54. The decline in RuBPCase-cm^2 could be caused by either a decline in the kinetics or in the amount of the enzyme. The seasonal trend of RuBPCase-Prot, which accounts for changes in the enzyme amount, follows no clear pattern. The regression of RuBPCase-Prot over days yielded a slope which did not differ from zero, indicating that RuBPCase-Prot was constant over time. Furthermore, sequential,orthogonal contrasts from day 25 onward indicated no changes in RuBPCase-Prot until day 51. Thus, because of these analyses, and that showing RuBPCase-cm^2 was correlated strongly with soluble protein ($r=0.82$), it can be concluded that the decline in RuBPCase activity was caused primarily by a loss of enzyme amount.

The RuBPCase-cm^2 activity in node-8 leaves was highly variable with time. A linear regression from day 46 to day

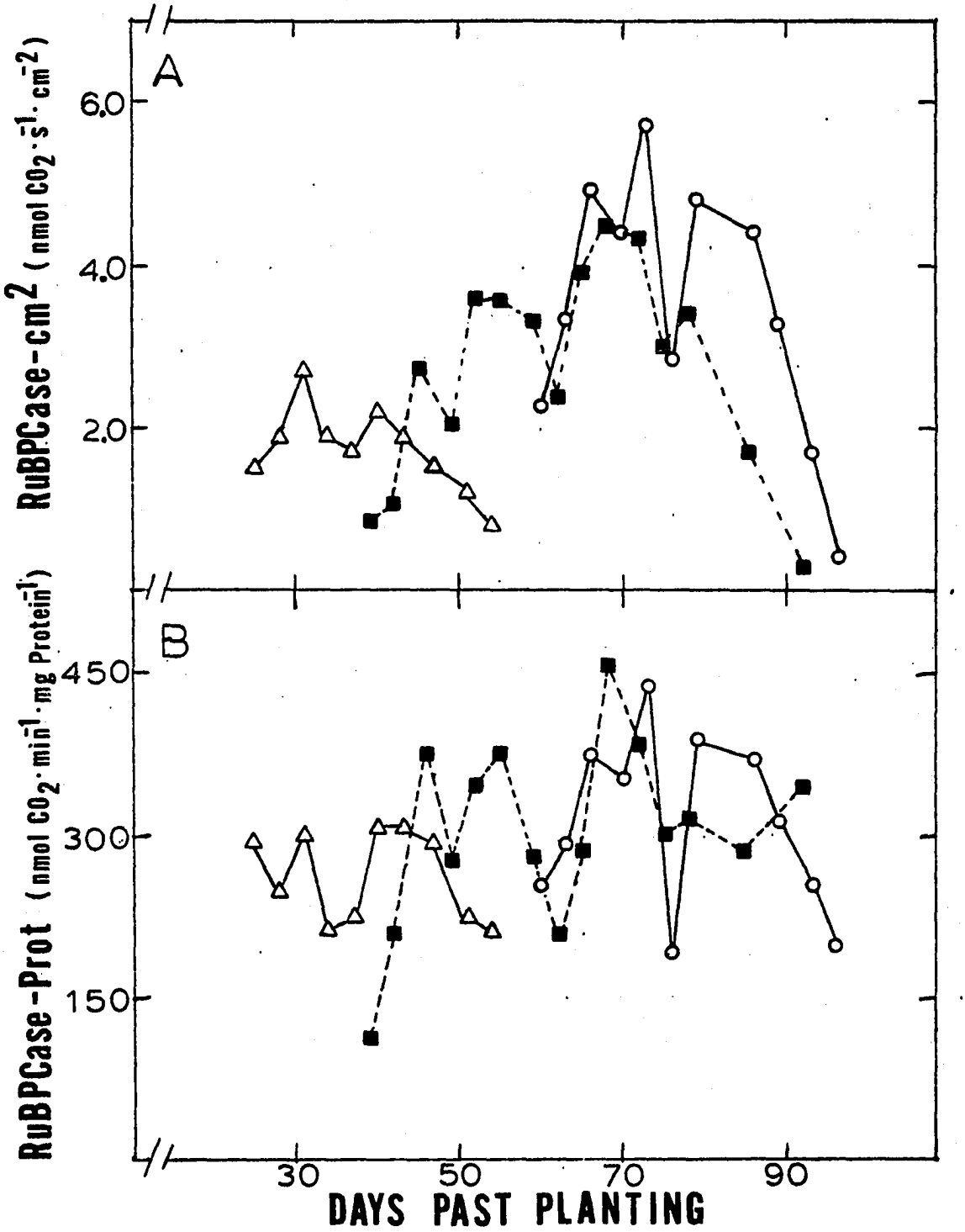


Figure 17. Seasonal trends of ribulose 1,5-bisphosphate carboxylase activity on a leaf area basis (RuBPCase-cm^2) (A) and on a soluble protein basis (RuBPCase-Prot) (B) at nodes 3 (△), 8 (■) and 13 (o)--1980

92 showed that the slope was equal to zero, and sequential, orthogonal contrasts indicated no significant changes in RuBPCase-Prot over the same period. Therefore, as in node-3 leaves, the decline in RuBPCase-cm^2 is a result of RuBPCase degradation.

In node-13 leaves, RuBPCase-cm^2 increased 114% from day 60 to day 73. With the exception of the seemingly deviant point on day 76, the apparent decrease from day 73 was continuous until the end of the sampling period. A sequential, orthogonal contrast (with day 76 data omitted) indicated that the first significant decrease occurred on day 89. Unlike the other two nodes, RuBPCase-cm^2 was highly correlated ($r=0.90$) with RuBPCase-Prot. But RuBPCase-cm^2 also was correlated strongly ($r=0.82$) with soluble protein content. These facts, coupled with the nonsignificant correlation between soluble protein and RuBPCase-Prot, indicate that both protein amount and enzyme kinetics are responsible for the changes in RuBPCase activity. From day 86 onward, protein declined 8.7% per day, RuBPCase-cm^2 declined 9.0% per day and RuBPCase-Prot declined 4.5% per day. Thus, it seems as though the loss of protein may have contributed more to the decline in RuBPCase-cm^2 activity than did the loss in kinetic properties.

Associations with CER

The following section is divided into subsections, each focusing on the relationships in leaves at a particular node. A day of decline has been defined for each parameter. This day of decline is the day after which a decline begins, and after which no statistically significant increase is observed. This does not mean that the day of decline and the subsequent sampling day are significantly different. For some parameters in which a gradual change occurs over time, it is difficult to separate statistically two adjacent points even though a declining trend is apparent or seems evident. The regression coefficients and the r^2 values in Tables 4 and 5 are calculated from the regression of CER on several traits during the senescence phase, that is, when CER is declining. The percentage rate of decline is a quantitative way of comparing changes between CER and the parameters during the senescence phase. It is calculated by dividing the regression coefficient by the predicted value of the parameter on the day of decline.

Node-3 leaves

The senescence stage in node-3 leaves begins after day 31 when CER clearly begins to decline. Table 4 presents some of the data calculated from the senescence stage in leaves at node 3.

Table 4. Summary statistics of some parameters during the senescence phase in node-3 leaves

Parameter[a]	Day of decline	Percentage rate of decline	Regression on CER: Regression coefficient	Regression on CER: r^2
CER	31	3.3**	-	-
CHL	31	1.6*	90 ± 28*	0.63
RuBPCase-cm^2	31	2.3**	1.1 ± 0.2**	0.82
RuBPCase-Prot	43	0.4	0.005 ± 0.006	0.11
Protein	34	2.4**	6.0 ± 1.0**	0.85
r_1	37[b]	2.9[b]	-1.1 ± 0.6	0.39

[a]Abbreviations as per Table 1.

[b]Incline.

*,**Indicate significantly different from zero at the 0.05 and 0.01 levels of probability, respectively.

From Table 4, it can be seen that two parameters, namely RuBPCase-Prot and r_1, are not associated well with CER during senescence. Both parameters begin to change after CER does and both have regression coefficients that are not different from zero, which implies that, really, they do not change with time. Moreover, they can account for only 11% and 39%, respectively, of the variation in CER. Although r_1 has a percentage rate of change most similar to CER, there is so much variability in the data that statistically the rate does not differ from zero. Therefore, neither RuBPCase-Prot nor

r_1 can be implicated as causes for the change in CER.

CHL and RuBPCase-cm^2 have a day of decline similar to CER, whereas, protein begins to decline 3 days after CER. However, it is RuBPCase-cm^2 and protein rather than CHL that have a percentage decline most similar to CER, as well as accounting for more variability in CER (82% and 85%, respectively) than CHL (63%). Nevertheless, CHL is seasonally coincident with CER, and cannot be ruled out as being a cause of decline.

Because protein and RuBPCase-cm^2 have a similar relationship to CER and because they are correlated well over the season ($r=0.82$) and during senescence ($r=0.83$), and furthermore, because RuBPCase specific activity does not change statistically throughout the season, the seasonal changes in RuBPCase-cm^2 can be attributed to changes in the amount of protein rather than enzyme kinetics. Thus, the two factors that are important in affecting CER during senescence of leaves at node 3 are: (1) protein or RuBPCase content and (2) chlorophyll content.

Node-13 leaves

Table 5 shows the summary statistics for leaves at node 13. The beginning of senescence in node-13 leaves occurred on day 86, and the data suggest that four parameters, namely CHL, RuBPCase-cm^2, RuBPCase-Prot and protein, are associated with the decline in CER. These four parameters have high r^2

Table 5. Summary statistics of some parameters during the senescence phase in node-13 leaves

Parameter[a]	Day of decline	Percentage rate of decline	Regression on CER: Regression coefficient	Regression on CER: r^2
CER	86	8.5*	-	-
CHL	86	7.6**	50.7 ± 5.9*	0.97
RuBPCase-cm^2	86	9.0**	0.52 ± 0.08*	0.95
RuBPCase-Prot	86	4.5**	0.124 ± 0.002**	0.97
Protein	86	8.7*	3.3 ± 0.7*	0.93
r_1	89[b]	81.2	-0.24 ± 0.10	0.75

[a]Abbreviations used as per Table 1.

[b]Increase.

*,**Indicate significantly different from zero at the 0.05 and 0.01 levels of probability, respectively.

values based on their regression on CER, have a day of decline similar to that of CER, have regression coefficients that are significantly different from zero and, excepting RuBPCase-Prot, have similar percentage rates of decline.

As mentioned previously, the relationships among RuBPCase-cm^2, RuBPCase-Prot and protein indicate that the loss in RuBPCase-cm^2 activity comes as a result more from the loss of the enzyme than from a change in kinetics. Thus, the decline in CER in node-13 leaves seems to be

affected both by a loss in the amount and, to a lesser extent, the kinetics of RuBPCase, as well as a decline in CHL.

Node-8 leaves

Node-8 leaf data are discussed last because the CER seasonal pattern is distinctly different from the seasonal patterns of the other two nodes. As previously mentioned, there are three stages of decline: stage 1, from day 52 to day 59; stage 2, from day 59 to day 78; and stage 3, from day 78 to day 92. These stages have daily rates of decline for CER of 3.7%, 0.5% and 5.6%, respectively.

None of the parameters studied showed trends similar to the three stages exhibited by CER. In fact, during stage 2, CHL, soluble protein and RuBPCase-cm^2 continued to increase as CER decreased slowly. Furthermore, since neither r_1 nor RuBPCase-Prot changed during much of the season, they seemed not to be associated with any stage of CER decline.

The only phenomenon that was associated with the change in CER from stage 1 to stage 2 was the beginning of rapid pod growth at node 8 (Figure 14A). The data suggest that CER begins to decline after day 52, then, as pods begin to gain weight rapidly, the rate of the decline in CER is retarded.

CHL, soluble protein and RuBPCase-cm^2 began to decline shortly before the stage-3 CER decline, and then declined similarly and linearly to CER during this period. The daily percentage decline in CER over this period was 5.6%, while

for soluble protein, RuBPCase-cm^2 and CHL, it was 6.6%, 6.7% and 5.3%, respectively. As mentioned earlier, the decline in RuBPCase-cm^2 in leaves at this node was attributable to the loss of protein rather than a change in kinetics. Thus, the final decline in CER was associated with similar declines in soluble protein or RuBPCase.

Comparisons Among Nodes

The final period of decline in CER in leaves at all nodes was accompanied by declines in CHL, soluble protein and RuBPCase activity. In leaves at nodes 3 and 8, the decline in RuBPCase activity was caused, presumably, by the degradation of RuBPCase, whereas in node-13 leaves, specific activity also declined, but not as rapidly as enzyme amount. All parameters declined most rapidly in node-13 leaves, and least rapidly in node-3 leaves.

Regression coefficients from the regression of CER on only those parameters that declined coincident with CER during the respective senescence periods in all leaves are presented in Table 6. A comparison among the nodes for a given parameter indicates that leaves at nodes 8 and 13 had similar regression coefficients, whereas the regression coefficients for leaves at node 3 were 78 to 141% greater. Thus, the quantitative relationships between CER and CHL, soluble protein and RuBPCase-cm^2 during senescence were similar between

Table 6. Regression coefficients from the regression of CER[a] on several parameters during senescence of leaves at nodes 3, 8 and 13

Node	n	CHL	Protein	RuBPCase-cm^2
3	8	90.3 ± 28.2[b]	5.95 ± 1.02	1.10 ± 0.21
8	3	49.5 ± 4.4	2.47 ± 0.17	0.46 ± 0.07
13	4	50.7 ± 5.9	3.31 ± 0.65	0.52 ± 0.08

[a]Abbreviations used as per Table 1.

[b]Standard error of the estimate.

leaves at nodes 8 and 13, but not at node 3.

Conclusion

The same parameters, namely soluble protein, CHL and RuBPCase-cm^2, decline coincident with CER in leaves at nodes 3 and 8. However, in addition to those three parameters, RuBPCase-Prot declined coincident with CER in leaves at node 13. The quantitative relationship between CER and CHL, soluble protein and RuBPCase-cm^2 during senescence was alike in nodes 8 and 13 leaves, but node-3 leaves differed. Because qualitative and quantitative differences exist during senescence among the nodes, it can be concluded that leaves of different plant ontogeny senesce differently.

GENERAL DISCUSSION AND CONCLUSION

The objectives of this study were to characterize metabolic trends between leaves of similar plant ontogeny and among leaves of different plant ontogeny, and then to determine which metabolic parameters were best associated with the decline in leaf photosynthesis. The data showed that leaves of different plant ontogeny had different seasonal metabolic patterns, and that the decline in CER was associated with declines in CHL and RuBPCase activity in all leaves.

Differences were observed among the leaves in the cause of the decline in RuBPCase activity. Leaves that emerged at nodes where pod growth was beginning showed a decline in RuBPCase attributable to degradation of RuBPCase and a change in enzyme specific activity, whereas in the other leaves, RuBPCase amount, but not specific activity, declined.

Although seasonal trends differed among leaves, the quantitative relationships between CER and several traits were similar for leaves that emerged after the beginning of flowering.

Leaf Photosynthesis in the Aging Plant

Leaf photosynthesis has a characteristic ontogenetic pattern that is influenced by factors both within the leaf (intrinsic factors) and elsewhere in the plant (extrinsic factors). Intrinsic factors include physical and biochemical

controls such as resistance to gas diffusion and enzymic regulatory systems. Among the more influential extrinsic factors are the assimilate-requiring sinks, such as newly emerged and growing leaves, roots, flowers and pods. These extrinsic factors may control leaf photosynthesis by serving as repositories for newly produced or stored assimilates, or they may act as sources of plant growth regulators that induce changes in leaf activity. Either way, these extrinsic factors effect changes within the leaf and, thus, are linked to intrinsic controls. The result of intrinsic and extrinsic factors over the life of a leaf is expressed in the ontogenetic trend of photosynthesis.

The ontogenetic trend of photosynthesis in leaves of a young plant is characterized by an early maximum followed by a gradual decline (Figure 15; Fraser and Bidwell, 1974; Woodward, 1976; Silvius et al., 1978). Leaves that emerge when plants have begun to flower but have not yet begun to fill pods display a photosynthesis pattern that is characterized by a decline shortly after the maximum photosynthesis is attained, then the rate of the decline is retarded as pods gain weight before resuming a faster rate of decline once the pods have neared full size (Figure 15; Woodward, 1976). Leaves that emerge on plants that have already entered the rapid pod growth period show a prolonged period, varying in time, of high photosynthesis before a very rapid decline

begins as the plant itself enters the final stages of senescence (Figures 8 and 15; Wittenbach et al., 1980).

There was circumstantial evidence that the extrinsic effect of pod growth influenced photosynthesis in leaves at nodes 8 and 13 in the second experiment, but not in leaves at either node sampled in the first experiment. If newly emerging leaves or roots had any effect, it was not evident in any of the data. This agrees with the findings of Fraser and Bidwell (1974) who concluded that "new leaves do not constitute major or long-lasting sinks." The effect of pods on photosynthetic rate has been well investigated (Woodward and Rawson, 1976; Nooden et al., 1978; Mondal et al., 1978) and will be discussed in a following section.

The strong and repeated relationship between CER and several traits during each leaf's senescence period suggests that photosynthesis is, to a large extent, controlled by or dependent on similar intrinsic factors in each leaf.

Factors Influencing Photosynthesis During Leaf Senescence

Extrinsic factors

The appearance and subsequent growth of pods at node 8, and perhaps at node 13, seemed to influence the seasonal pattern of leaf photosynthesis. The presence of pods has been shown to maintain high rates of photosynthesis (Lawn and Brun, 1974; Woodward and Rawson, 1976; Mondal et al., 1978) as well

as to initiate monocarpic senescence (Nooden et al., 1978). The maintenance of high photosynthesis by the presence of pods has been attributed to the alleviation of end product inhibition, promotion of chloroplastic starch degradation, and seed-originated hormone signals (Mondal et al., 1978). On the other hand, these same reasons form the basis for the various hypotheses regarding the role of the pods in monocarpic senescence. Briefly, the two major hypotheses that have been proposed to explain the mechanism by which the pods induce monocarpic senescence are: (1) that they divert nutrients going to or drain nutrients away from the leaves; and (2) that the pods, actually the seeds, are the source of a "death" hormone (Nooden et al., 1978).

The coincidence of the beginning of rapid pod growth with the retardation in the decline of CER in node-8 leaves (Figures 14A and 15A) is readily apparent. The maintenance of high photosynthetic rate during rapid pod growth is also evident in node-13 leaves. CER in both leaves begins to decline at nearly the same time, which also seems true for leaves at nodes 12 and 15 (Figure 8) in the previous year. This synchronous decline may be the initiation of monocarpic senescence--the "death" signal. Nooden et al. (1978) predict that monocarpic senescence begins when the dry weight of the seeds is approximately 90% of maximum. At nodes 8 and 13 (1980), this corresponds approximately to 80 days after plant-

ing, the time when CER began to decline precipitously. But the role of pods as the inducers of senescence is tenuous. At nodes 12 and 15 (1979), 90% of maximum pod weight occurred about 15 days after CER had begun to decline rapidly. The role of the pods (seeds) in causing the decline of photosynthesis can be further questioned. In studies where pods have been removed from plants, photosynthesis has been shown to decline, not only at the same rate, but at the same time as podded, control plants (Woodward and Rawson, 1976; Mondal et al., 1978). Therefore, although pods may maintain high photosynthesis, their role in senescence is equivocal, and it seems as though intrinsic factors may play the leading role.

Intrinsic factors

The intrinsic factors found to be common to the decline in CER, or leaf senescence, in this study are CHL, protein and RuBPCase activity.

Chlorophyll The loss of chlorophyll is certainly one of the easiest methods employed to define and characterize leaf senescence. Much, if not all, of the work on monocarpic senescence by Nooden and his coworkers is based on leaf yellowing as an index of senescence. There is no doubt that chlorophyll is functionally linked to photosynthesis, but changes in its content may not be an accurate indication of changes in photosynthesis. In the depodding studies of

Mondal et al. (1978), leaf chlorophyll content remained high even though photosynthesis declined. A nonyellowing mutant of meadow fescue has been shown to undergo senescence similar to the normal plant, except that chlorophyll does not degrade.

There is evidence that a surplus of chlorophyll exists in leaves. Silvius et al. (1978) reported that chlorophyll content in the second trifoliolate soybean leaf continued to increase after CER reached a maximum. A similar relationship was reported in leaves "at a node about two-thirds of the way up" the soybean plant (Wittenbach et al., 1980). A decline in chlorophyll content preceding the decline in photosynthesis has been reported in soybean leaves (Sesay and Shibles, 1980) and in wheat leaves (Feller and Erismann, 1978; Hall et al., 1978). That, in some cases, chlorophyll content continues to increase after photosynthesis ceases to increase, and that, in other cases, chlorophyll content begins to decline before photosynthesis begins to decline, implies that chlorophyll content may not be tightly coupled to photosynthesis.

My results show a close relationship between chlorophyll content and photosynthesis in time. In general, CHL began to decline coincident with and at a slower rate, except in node-8 leaves, than CER during each leaf's period of senescence. Whether chlorophyll is a causal factor in leaf senescence, however, is moot.

Protein content Because Wittenbach and his coworkers (1978, 1980) observed interference with the Lowry et al. (1951) method of analysis, especially during leaf senescence, they recommended the use of the Bio-Rad (1977) procedure for estimating protein content. They attributed the discrepancies between their results (1980) and those of Mondal et al. (1978) to the difference in analytical methods employed. In my second experiment, I used both methods until the Lowry method, for some unexplained reason, no longer functioned well. As mentioned in the Part II Materials and Methods, until that time, there was a high correlation between the two methods--before the final senescence period began.

Another plausible explanation for the discrepancies observed between the results of Mondal et al. (1978) and Wittenbach et al. (1980) is the difference in leaf sampling methods employed. Whereas Wittenbach et al. attempted to sample leaves at a particular node, Mondal et al. used what I call a sequential, acropetal sampling (SAS) procedure. The SAS procedure consists of selecting a leaf to be sampled on the basis of its position relative to the apex. As long as the plant continues to elongate and produce new leaves, each sampled leaf continues to be of the same or nearly the same age. Thus, age of the sampled leaf remains constant through the sampling period until leaf production ceases, after which the effect of leaf aging is manifested. The SAS results in a

seasonal trend characterized by a plateau followed by a decline (Mondal et al., 1978; Sesay and Shibles, 1980).

The ontogenetic patterns for crude protein at nodes 12 and 15 in 1979 (Figure 11A) somewhat resemble those reported by Mondal et al. (1978); that is, little change over the season. It is not known, though, whether the pattern was a consequence of using the Lowry method, because no comparisons were made with the Bio-Rad procedure for leaves of that plant ontogeny. Wittenbach et al. (1980) reported that in their work the Lowry procedure gave results similar to Mondal et al., but since the results did not agree with those of Kjeldahl N analysis, they discontinued using it.

The decline in Bio-Rad-assayed soluble protein content was similar to the respective CER decline in leaves at each sampled node. Generally, protein content declined within 3 days of the decline in CER. Wittenbach et al. (1980), on the other hand, observed that the decline in protein content in soybean leaves followed the decline in photosynthesis by almost 2 weeks. Yet, in cereal leaves, the decline in photosynthesis is preceded by a decline in soluble protein (Wittenbach, 1979; Friedrich and Huffaker, 1980). A possible explanation for the discrepancy between Wittenbach et al. (1980) and the other reports, including this one, is that Wittenbach et al. sampled leaves at "the same approximate node", whereas in the other reports, investigators sampled

leaves at a specific position on the plant. Nevertheless, the degradation of protein during the senescence period implies that protease activity has increased.

Proteolysis is recognized as one of the first major features in leaf senescence (Thimann, 1978; Peoples and Dalling, 1978; Wittenbach et al., 1980). Attempts have been made to correlate protein decline with protease activity in leaves and, in some cases, a correlation was found (Peterson and Huffaker, 1975; Feller et al., 1977; Wittenbach, 1979), whereas in others, a relation between protein amount and proteolytic activity was not observed (Anderson and Rowan, 1965; Ragster and Chrispeels, 1979; Friedrich and Huffaker, 1980). The various substrates and methods employed to assay protease activity can account for some of the discrepancies (Ragster and Chrispeel, 1981a). Nevertheless, the results of investigations with soybean leaves demonstrate that RuBPCase-digesting proteases are present in the cell cytoplasm, and that an increase in the activity of one or more of these proteases occurs during leaf senescence (Wittenbach et al., 1980; Ragster and Chrispeels, 1981b). No accumulation of amino-N is observed during the period of protein degradation in leaves of late plant ontogeny, thus indicating a rapid transport of amino acids from the leaf (Wittenbach et al., 1980). To my knowledge, no studies have been performed to determine the fate of amino-N liberated from protein during the senescence of leaves from young soybean plants. It is possible that the

pods present during the senescence of upper leaves act as a strong N sink, thus accounting for the faster rate of protein degradation observed in those leaves, whereas in the lower leaves an accumulation of amino acids may exert some feedback control on protease activity. Alternatively, the amino-N may be exported to active vegetative sinks.

RuBPCase The key photosynthetic enzyme, and the most abundant of all plant proteins, RuBPCase, constitutes about 50% of the soluble protein over the life of a soybean leaf (Wittenbach et al., 1980). It is a large enzyme having a molecular weight of about 550,000, consisting of eight large and eight small subunits. The enzyme is synthesized cooperatively by the chloroplast genome, which codes for the catalytic, large subunit, and by the nuclear genome, which codes for the regulatory, small subunit (Jensen and Bahr, 1977). During leaf senescence, RuBPCase is preferentially degraded, accounting for up to 85% of the loss in soluble protein (Peoples and Dalling, 1978; Wittenbach, 1979; Friedrich and Huffaker, 1980; Wittenbach et al., 1980). A preferential loss of RuBPCase ought to result in a concurrent decline in photosynthesis.

There is a good correlation in several crop species between the decline in photosynthesis associated with senescence and the decline in RuBPCase activity (Hall et al., 1978; Peoples and Dalling, 1978; Wittenbach, 1979; Friedrich and

Huffaker, 1980). Hall et al. (1978) reported that the decline in RuBPCase activity was attributable to a decline in specific activity rather than a loss of enzyme amount. Using the same crop species, wheat, Wittenbach (1979) found that the decline in RuBPCase activity was not attributable to a loss of specific activity but, rather, to the amount of the enzyme. Wittenbach could not resolve the discrepancies between the two reports, but he did not consider that Hall et al. used primary wheat leaves whereas he used flag wheat leaves. The data presented in this paper show that the decline in RuBPCase activity in leaves that emerged on young or middle-aged plants was due to a decline in enzyme quantity, whereas for leaves emerging later, the decline is a composite of a decline in enzyme amount and specific activity. Thus, plant ontogeny may be an important factor that influences metabolic changes in each leaf's senescence.

Another discrepancy exists in the literature regarding the association between RuBPCase and photosynthesis. Wittenbach et al. (1980) reported that RuBPCase activity was not closely correlated with changes in photosynthesis, but did closely parallel change in total soluble protein. Thus, specific activity was constant, which agrees with my data for leaves at a node similar to the one they measured. But I found that RuBPCase activity was correlated very well with the decline in CER, which agrees with the senescence data of

Mondal et al. (1978). However, Mondal et al. (1978) observed no decline in protein coincident with the decline in photosynthesis and carboxylase activity associated with senescence. As previously mentioned, Mondal et al. (1978) used the Lowry method to determine protein, which, as mentioned earlier, may be an inaccurate method to estimate protein in soybean leaves.

That Wittenbach et al. (1980) observed a poor association between the decline of photosynthesis and RuBPCase-cm^2, whereas I observed a good association, is perplexing. They recognized that, during senescence, carboxylase activity and amount did parallel the decline in photosynthesis, but that the onset of the decline did not appear to be tightly coupled as it was in wheat. Because of the tight coupling between the two declines in wheat, they mentioned that they were further studying the onset of the declines in soybean. The data presented in this dissertation indicate that the onset of the declines is tightly coupled.

The Senescence Program

Senescence is an inherent, programmed event in the life of a leaf and, thus, is a regulated event. A series of events occur that lead to the eventual death of the leaf. The beginning of senescence is clearly signaled by the onset of the decline in photosynthesis, the primary function of the

leaf. Because photosynthesis is a complex process involving physical and biochemical factors, it is difficult, if not impossible, to pin-point the exact cause or time of its decline. Yet, there is evidence that the degradation of the chloroplast is an event closely related to the onset of senescence.

There is sufficient evidence in the literature, according to Thomas and Stoddart (1980), to indicate that, by the time a leaf has reached maturity, the chloroplast genome has become almost completely repressed. The repression of the genome results in no more nucleic acids being synthesized or replicated. Without new nucleic acids, those remaining are responsible for the coding and synthesis of chloroplast protein, for example, the large subunit of RuBPCase. When the existing nucleic acids become degraded, essentially all chloroplast-dependent protein synthesis stops. Then the integrity of the chloroplast will decline. Batt and Woolhouse (1975) have demonstrated that the decline in chloroplast enzymes precedes the decline in cytoplasm enzyme activity. Moreover, among the first symptoms of senescence is a change in the characteristics of the chloroplast. The chloroplast envelope has been shown to separate its inner and outer membranes (Thomas and Stoddart, 1980). Also noted has been a swelling of the chloroplast accompanied by a disorientation of the chloroplast lamellae (Wittenbach et al., 1980).

Because young and mature soybean leaves have sufficient protease activity to degrade RuBPCase in relatively short periods of time, the proteases must be separate from their substrate (Ragster and Chrispeels, 1981b). During the time when the chloroplast envelope is undergoing a separation, it is likely that its transport characteristics are also changing. Because the RuBPCase-attacking proteases are not found in the chloroplast (Ragster and Chrispeels, 1981b), they either must enter the chloroplast or the substrate must leave the chloroplast. Thus, proteolysis is not controlled by protease activity but rather through compartmentalization. This separation of protein and protease may be a key step in the senescence program.

During soybean leaf senescence, the number of chloroplasts per cell has been observed to decline (Wittenbach et al., 1980). In Brassica sp. (L.), chlorophyll content per chloroplast is not related to photosynthesis, whereas an increase in chloroplast number per unit leaf area is related to an increase in the rate (Kariya and Tsunoda, 1972). These facts support my contention that chloroplast degradation is a major factor in leaf senescence. The differences in senescence patterns among leaves may very well be related to chloroplast number per leaf area or the rate of chloroplast degradation.

In those leaves where a decline in RuBPCase specific activity was observed, it is possible that proteases specific

or preferential to the regulatory, small subunit are more active or abundant. Furthermore, leaves of different plant ontogeny may have different metabolite balances that can influence the rate of chloroplast metabolism and degradation.

In summary, I propose that differences in the senescence pattern among leaves are partly accounted for by differential rates of chloroplast degradation, and that extrinsic and intrinsic factors may influence the rate of chloroplast degradation, but that the onset of senescence of a leaf is an intrinsic event.

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APPENDIX

Table A1. Mean squares from analysis of variance and node means for some physiological characteristics[a]--1979

Source	df	Area	SLW	SFW	Pod[b]
Regression on days	2				
Linear	1	69.5	19.73**	71.15**	2301.42**
Quadratic	1	90.7	31.80**	20.62**	11.97
Node	1	2.4	9.60**	136.63**	86.29**
Node*linear	1	1728.5**	0.11	0.24	64.94**
Node*quadratic	1	222.4*	1.71*	5.16	28.88
Lack of fit	12	115.3*	0.51	4.21	1.22
Error		42.2	0.33	2.64	9.21
Error df		54	54	51	51
Total sums of squares		5779.9	87.09	418.9	2978.09
Node means					
Node 12		46.2	4.64	15.8	877
Node 15		45.3	5.69	17.5	675

[a]Abbreviations used: Area = leaflet area (cm^2), SLW = specific leaf dry weight (mg dry weight$\cdot cm^2$), SFW = specific leaf fresh weight (mg fresh weight$\cdot cm^2$), Pod = pod dry weight (mg dry weight), r_1 = leaf diffusive resistance to water ($s \cdot cm$), CER carbon dioxide exchange rate (nmol $CO_2 \cdot s^{-1} \cdot cm^{-2}$), Rn = leaf respiration rate (nmol $CO_2 \cdot s^{-1} \cdot cm^{-2}$), CHL = chlorophyll content ($mg \cdot cm^{-2}$), Prot = protein content ($mg \cdot cm^{-2}$), RuBPCase-Prot = carboxylase activity on protein basis (nmol $CO_2 \cdot s^{-1} \cdot$ mg protein^{-1}), RuBPCase-cm^2 = carboxylase activity on leaf area basis (nmol $CO_2 \cdot s^{-1} \cdot cm^{-2}$).

[b]x 10^4 to calculate actual mean squares.

[c]x 10^{-4} to calculate actual means squares.

*,**Indicate significance at the 5% and 1% probability levels, respectively.

r_1	CER	Rn	CHL[c]	Prot	RuBPCase -Prot	RuBPCase -cm^2
77.17**	5.094**	0.0128*	37.21**	0.221	24.53**	38.54**
38.34**	14.407**	0.0783**	58.88**	5.434**	11.23**	86.19**
0.00	1.424**	0.0400**	11.23**	0.852**	0.93*	8.18**
0.02	0.236	0.0014	0.07	0.585*	0.37	0.01
2.72	2.648**	0.0060	0.61	0.221	0.15	3.98**
2.42*	0.133	0.0077**	1.67**	0.176*	1.25**	2.80**
1.03	0.071	0.0019	0.43	0.084	0.13	0.46
54	54	54	54	51	49	52
202.91	29.409	0.3327	151.50	13.699	58.75	194.35
2.15	1.38	0.204	0.0487	1.58	2.02	3.25
2.12	1.75	0.259	0.0580	1.93	2.24	4.13

Table A2. Means of each day and L.S.D.s (.05) for some physiological characteristics[a] of leaves at nodes 12 and 15--1979

Day	Area	CER	Rn	CHL	Prot	SLW	RuBPcase-cm^2	RuBPCase-Prot	r_1	Leaf
					Node 12					
66	43.80	1.33	0.211	0.0441	1.32	2.97	1.93	1.49	1.88	2.75
70	49.76	1.55	0.219	0.0580	1.59	3.85	3.41	2.19	0.83	4.00
73	39.04	1.50	0.183	0.0485	1.44	4.21	3.89	2.77	1.17	3.50
76	48.19	1.76	0.242	0.0629	1.48	4.31	5.37	3.61	1.32	4.25
79	41.86	1.63	0.192	0.0557	1.78	5.14	4.83	2.78	1.88	3.75
86	44.54	1.93	0.257	0.0561	1.72	5.20	4.13	2.26	1.62	4.50
91	40.47	1.64	0.203	0.0546	1.88	5.58	3.14	1.69	1.97	4.00
97	46.99	1.19	0.235	0.0430	1.97	5.20	2.86	1.43	2.34	4.75
104	48.28	0.96	0.204	0.0504	1.78	5.36	2.59	1.47	2.71	5.00
109	59.19	0.26	0.099	0.0141	0.87	4.58	0.51	0.53	5.80	5.50
L.S.D. .05	10.06	0.30	0.061	0.0073	0.40	0.77	0.89	0.54	1.39	.754
					Node 15					
71	47.55	1.42	0.218	0.0562	1.31	3.64	3.75	3.14	1.31	3.25
74	55.35	1.87	0.309	0.0603	1.64	5.31	4.93	3.09	0.94	5.25
78	53.94	1.67	0.212	0.0738	2.03	6.07	4.26	2.16	2.22	5.50
85	53.61	2.63	0.370	0.0690	2.05	6.49	7.13	3.48	1.37	6.25
90	36.87	2.55	0.246	0.0681	2.07	6.04	4.59	2.32	1.27	5.00
95	44.99	2.16	0.295	0.0568	2.42	6.73	5.08	2.16	1.49	5.25
102	30.91	1.23	0.250	0.0438	2.19	5.76	2.10	0.93	2.34	4.00
107	38.92	0.45	0.174	0.0367	1.48	5.50	1.54	1.04	6.06	5.75
L.S.D. .05	8.57	0.47	0.065	0.0118	0.43	0.92	1.06	0.48	1.57	0.77

[a]Abbreviations as per Table 1.

Table A3. Mean squares from analysis of variance for regression on days of some physiological characteristics[a] for nodes 12 and 15--1979

Source	df	SLW	Rn	CER	CHL	Prot	RuBPCase -cm^2	RuBPCase -Prot	r_1
Node 12									
Linear	1	11.02**	0.0128**	3.75**	0.00227**	0.0014	19.05**	11.72**	42.98**
Quadratic	1	11.76**	0.0252**	4.14**	0.00284**	2.4214**	38.17**	7.36**	16.71**
Lack of fit	7	0.17	0.0043	0.06	0.000231**	0.1920*	2.11**	1.29**	1.49
Error		0.28	0.0018	0.04	0.000026	0.0761	0.38	0.14	0.93
Error	df	30	30	30	30	29	30	29	30
Node 15									
Linear	1	5.10**	0.0063	2.16**	0.00204**	0.252	25.55**	15.68**	35.61**
Quadratic	1	15.39**	0.0408**	11.29**	0.00210**	2.590**	41.43**	1.67**	22.93**
Lack of fit	5	1.00*	0.0126**	0.24	0.000078	0.155	3.77**	1.20**	3.76*
Error		0.39	0.0020	0.11	0.000066	0.094	0.57	0.13	1.15
Error	df	24	24	24	24	22	22	20	24

[a]Abbreviations as per Table 1.

*,**Indicate significance at the 5% and 1% probability levels, respectively.

Table A4. Mean squares from analyses of variance and node means for some physiological characteristics[a] expressed on a dry weight basis--1979

Source	df	CER	Rn	CHL	RuBPCase-DW	Prot
Regression on days	2					
Linear	1	629878**	5645.1**	597.68**	3645972**	58771**
Quadratic	1	106716**	28.3	2.68	1056040**	3414
Node		1101	152.9	0.58	25024	1927
Node*linear	1	16884*	85.5	1.29	94	35405**
Node*quadratic	1	36045**	6.4	4.35	304	1475
Lack of fit	12	9801*	353.7*	10.63	106606**	7717*
Error		2398	62.5	3.22	8722	1736
Error df		54	54	54	52	51
Total sums of squares		1037715	13536.8	908.11	6460250	282063
Node means						
Node 12		307	45.5	10.9	714	347
Node 15		308	46.3	10.5	745	331

[a]Abbreviations used: CER = carbon dioxide exchange rate (nmol $CO_2 \cdot s^{-1} \cdot g$ dry $weight^{-1}$); Rn = respiration rate (nmol $CO_2 \cdot s^{-1} \cdot g$ dry $weight^{-1}$); CHL = chlorophyll concentration ($mg \cdot g$ dry $weight^{-1}$); RuBPCase-DW = carboxylase activity (nmol $CO_2 \cdot s^{-1} \cdot g$ dry $weight^{-1}$); Prot = protein concentration ($mg \cdot g$ dry $weight^{-1}$).

*,**Indicate significance at the 5% and 1% probability levels, respectively.

Table A5. Mean squares from combined analysis of variance, and means and L.S.D.s for several parameters--1980

Source	df	Area[a]	SLW	r_1	CHL	Prot	CER	RuBPCase -cm^2	RuBPCase -Prot
Nodes	2	45081**	25.96**	1.30	0.00213**	0.661**	2.38**	33.87**	39632**
Days (node)	33	381**	0.60**	4.75**	0.00038**	0.118**	1.86**	5.76**	23352**
Error	107	40	0.14	1.36	0.00003	0.009	0.10	0.48	3584
Means									
Node 3		34.8	3.12	1.38	0.0301	0.392	1.76	1.73	262
Node 8		79.9	3.66	1.59	0.0353	0.513	1.82	2.72	314
Node 13		24.8	4.66	1.28	0.0442	0.644	2.19	3.54	317
L.S.D.$_{(0.05)}$		2.9	0.17	ns[b]	0.0025	0.043	0.14	0.32	27

[a]Abbreviations as per Table 1.

[b]ns = nonsignificant.

**Indicates significance at the 1% probability level.

Table A6. Means for each day and the L.S.D. for come parameters in node-3 leaves--1980

Day	Leaf[a]	Area	Pod	SLW	r_1	CER	CHL	Prot	RuBPCase -cm^2	RuBPCase -Prot[b]
25	2.75	33.3	0	2.72	0.85	2.38	0.0208	0.293	1.51	297
28	4.00	33.3	0	3.34	0.91	2.60	0.0365	0.467	1.94	248
31	5.00	35.6	0	3.36	1.00	2.71	0.0381	0.528	2.67	300
34	5.75	37.3	0	3.25	1.28	2.23	0.0291	0.528	1.86	211
37	6.00	33.8	0	3.44	1.01	1.69	0.0357	0.464	1.75	224
40	7.50	34.4	0	2.97	2.08	1.54	0.0343	0.416	2.16	308
43	7.75	37.1	0	2.92	1.62	1.81	0.0335	0.357	1.88	309
47	9.50	31.8	5.5	2.98	1.74	1.12	0.0254	0.312	1.50	289
51	11.00	37.1	18.5	3.15	1.53	0.91	0.0277	0.315	1.22	224
54	11.50	34.4	1.5	3.04	1.78	0.62	0.0199	0.237	0.82	216
L.S.D. .05	0.71	7.17	5.15	0.36	0.59	0.40	0.0053	0.114	0.78	65

[a]Abbreviations as per Table 1.

[b]nmol $CO_2 \cdot min^{-1} \cdot mg\ protein^{-1}$.

Table A7. Means for each day and the L.S.D. for some parameters in node-8 leaves--1980

Day	Leaf[a]	Area	Pod	SLW	r_1	CER	CHL	Prot	RuBPCase -cm^2	RuBPcase -Prot[b]
39	2.00	32.1	0	3.00	2.82	0.86	0.0237	0.469	0.86	111
42	3.00	71.8	0	2.75	1.42	1.99	0.0222	0.319	1.10	207
46	4.00	77.4	16.8	3.23	1.32	2.28	0.0365	0.431	2.74	380
49	5.00	83.2	33.0	3.43	1.14	2.59	0.0358	0.457	2.11	279
52	6.00	80.0	43.0	3.64	1.01	2.65	0.0416	0.620	3.60	348
55	6.75	76.9	154.8	3.58	2.10	2.25	0.0395	0.578	3.55	378
59	8.00	83.0	234.8	3.87	1.66	1.96	0.0441	0.710	3.36	280
62	8.50	85.1	364.0	3.97	1.38	2.05	0.0460	0.680	2.40	211
65	9.25	87.9	519.5	4.04	1.68	1.72	0.0410	0.536	3.91	435
68	9.25	91.1	735.0	4.03	1.71	1.87	0.0384	0.594	4.48	455
72	9.50	86.5	857.3	4.18	1.12	2.06	0.0455	0.678	4.34	382
75	10.00	88.3	976.5	3.96	1.57	1.63	0.0418	0.583	3.01	299
78	10.50	95.5	1481.5	4.35	0.62	1.83	0.0371	0.634	3.40	318
85	8.50	80.9	1962.5	3.71	1.26	1.24	0.0277	0.360	1.69	284
92	9.25	78.8	1696.3	3.22	3.01	0.39	0.0083	0.046	0.27	340
L.S.D. .05	1.08	11.1	432.9	0.52	1.31	0.45	0.0063	0.131	1.01	94

[a]Abbreviations as per Table 1.

[b]nmol $CO_2 \cdot min^{-1} \cdot mg\ protein^{-1}$.

Table A8. Means for each day and the L.S.D. for some parameters in node-13 leaves--1980

Day	Leaf[a]	Area	POD	SLW	r_1	CER	CHL	Prot	RuBPCase -cm^2	RuBPCase -Prot[b]
60	2.75	17.9	76.5	3.96	1.13	2.52	0.0401	0.512	2.27	266
63	3.75	25.6	250.8	4.74	0.97	2.50	0.0455	0.698	3.35	291
66	3.25	23.4	432.0	5.02	1.21	2.80	0.0493	0.789	4.86	370
70	4.00	26.7	592.0	4.79	1.01	2.88	0.0501	0.751	4.41	356
73	3.25	24.6	744.8	4.65	0.36	2.59	0.0506	0.775	5.68	443
76	3.25	22.3	977.0	4.97	0.64	2.29	0.0478	0.894	2.88	191
79	4.00	27.6	1028.0	4.82	0.48	2.48	0.0523	0.747	4.81	388
86	3.50	23.8	1544.5	4.97	0.73	2.44	0.0534	0.698	4.40	372
89	3.75	27.3	1551.5	4.93	0.63	1.66	0.0449	0.625	3.31	317
93	4.50	31.8	1810.0	4.42	0.94	1.28	0.0322	0.359	1.74	258
96	4.00	20.0	1786.0	3.83	7.48	0.20	0.0116	0.102	0.43	201
L.S.D. .05	0.97	7.5	300.4	0.68	2.58	0.52	0.0089	0.151	1.14	92

[a]Abbreviations as per Table 1.

[b]nmol $CO_2 \cdot min^{-1} \cdot mg\ protein^{-1}$.