PLASTOCHRON INDEX – AN INDICATOR OF PLANT STRUCTURE AND FUNCTION. A CASE STUDY USING PISUM SATIVUM L.

THESIS

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IN MEMORY OF MY BELOVED FATHER,

ISAIAH OLUFUNBI OMOLOKUN

(1938 – 1998)

Who sacrificed a PhD in UK to take care of his children.

Daddy, I did not get to go to UK but I did the PhD you wanted.

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Definition of Symbols and Abbreviations

Abbreviation	**Definition**
PI	plant plastochron index
LPI	leaf plastochron index
DAS	days after sowing
RGR	relative growth rate
LAR	leaf area ratio
LAI	leaf area index
CO_2	carbon dioxide
$[CO_2]$	carbon dioxide concentration
N	nitrogen
[N]	nitrogen concentration
E	elevated CO_2
A	ambient CO_2
R	nodulated
r	non-nodulated
N	nutrient solution containing mineral nitrogen
n	nitrogen free-nutrient solutions
REn	nodulated plants supplied with N-free nutrient solution grown under elevated CO_2

RAn	nodulated plants supplied with N-free nutrient solution grown under ambient CO_2
REN	nodulating plants supplied with N+ nutrient solution under elevated CO_2
RAN	nodulating plants supplied with N+ nutrient solution ambient CO_2 (RAN)
rEN	non- nodulating plants supplied with N+ nutrient solution under elevated CO_2
rAN	non- nodulating plants supplied with N+ nutrient solution under ambient CO_2
RLA	rate of leaf appearance
LfER	leaflet elongation rate
SE	standard error
CFDA	carboxyfluorescein diacetate
CF	carboxyfluorescein
SE-CC	sieve element-companion cell

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FOR THE GREAT THINGS HE HAS DONE.

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Abstract

The use of chronological age for example, using days after sowing (DAS), or days after germination (DAG) as a time variable may result in the inherent variability between plants resulting in differences which can be large enough to obscure subtle developmental trends that become evident among plants sown at the same time. An alternative to DAS or DAG is the plastochron index (PI), first used by Erickson and Michelini (1957) as a morphological time scale and numerical index; which to according to the authors suggested and represented a more accurate reflection of the developmental status of a plant.

The research presented in this thesis was therefore aimed specifically at utilizing the index in qualitative and quantitative analyses, to confirm its usefulness in analyzing and predicting plant growth and development. Specifically this research focused on investigating various morphological and physiological events that together, hopefully, would serve as a template for the prediction of the growth, development and reactions of *Pisum sativum* L. to different growth conditions.

In Chapter 3, the use of the average length of the first pair of leaflets on each node as a suitable parameter for calculating PI in *P. sativum* is suggested. The results presented in Chapter 3 suggest that plant age is

best expressed using the plastochron index, as this reflects the time interval between the initiations of successive pairs of leaflets. This section of the research has been published as "Ade-Ademilua OE, Botha CEJ (2005) A re-evaluation of plastochron index in peas - a case for using leaflet length. South African Journal of Botany 71: 76-80".

The PI formula developed was subsequently used in this research to conduct qualitative and quantitative investigations of plant growth and development in which all data and observations were related directly to the plastochron index.

In Chapter 4, the sink to source transition in *Pisum sativum* L. leaves at different plastochron ages in nodulating plants was investigated using the phloem-mobile fluorescent marker, 5,6-carboxyfluorescein (5,6-CF). The results demonstrated that young leaves remained strong sinks up until LPI 0, after which sink-source transition occurred up to LPI 1.8 and leaflets transitioned to strong source systems by LPI 2.0.

A well-developed cross-connected phloem system between paired leaflets in peas, and the petiole and the stem vascular supply was observed. The data presented in the second part of Chapter 4 suggest that the phloem transport between leaflet pairs is independent of the sink/source state of the leaflets, or of movement along the source to sink gradient. The data support the presence of a modular transport

system which may ensure re-allocation and balancing between leaflets of the same physiological age and photosynthetic and transport status, thereby load-balancing the local transport system, before exporting to other younger (sink) regions.

The investigation of leaf development using the plastochron index (Chapter 5) revealed that the formation of air spaces in the palisade and spongy mesophyll, one of the preparatory events for transition from sink to source state in developing leaves, occurs between LPI 0 and LPI 1 in pea leaflets.

Results of the anatomical and ultrastructural study related to PI are presented in Chapter 5. The density of wall ingrowths in transfer cells of minor veins increased with LPI and appeared to be associated with the probable transition to source state and the related potential increase in the production of assimilates for export. The onset of wall ingrowth development in leaflets at LPI 0 provided evidence that sink-to-source transition commences at LPI 0 in *P. sativum*. Presumably-functional plasmodesmata as well as a few mature sieve elements were evident in class IV veins in the apical region of young and older leaflets at LPI 0. The number of mature sieve elements per vein however, increased with increasing LPI. Most class V veins were still undergoing division at LPI 0 and their sieve elements did not show signs of maturity until LPI 1. The increase in the number of mature metaphloem sieve elements in

young, supposedly importing tissue at LPI 0 to older, supposedly exporting tissues at LPI 2 is evidence of the association between phloem maturation and transition from importing to exporting status.

In Chapter 6, I report on the effects of elevated CO_2 on the growth and leaf development of nodulating and non-nodulating *Pisum sativum* L var. Greenfeast grown under controlled environment of the same nitrogen (6mM) and nitrogen-free nutrient solution conditions. Short-term exposure to elevated CO_2 induced rapid plant growth, irrespective of treatment. However, long-term elevated CO_2 treatment did not affect rate of leaf appearance (RLA) in nodulated plants, irrespective of mineral N supply but enhanced RLA in non-nodulating plants supplied with mineral N. Supplied N resulted in a significant increase in leaflet elongation rate (LfER) under both ambient and elevated CO_2, but LfER was not significantly affected by nodulation but was increased by high CO_2. This suggested that the growth of nodulating *P. sativum* L may not be significantly affected under CO_2 levels as high as 1000 µmol mol^{-1}. The data suggest that elevated CO_2 will enhance canopy size, provided adequate soil N is available and more so in non-nodulating plants. This section of the research has been published as "Ade-Ademilua OE, Botha CEJ (2004) The effects of elevated CO_2 and nitrogen availability supersedes the need for nodulation in peas grown under controlled environmental conditions. South African Journal of Botany 70: 816 – 823".

This thesis demonstrates that the similarity in the qualitative analyses results obtained from plants from different CO_2, nitrogen and nodulation treatment conditions, highlights the fact that plants of same PI value are at the same developmental state, irrespective of the growth condition. Furthermore, changes in plant structure and function observed under different growth conditions can be related simply to changes in plastochron index.

The work presented in this thesis demonstrate that changes in plant structure and function analyzed are related to changes in PI. An important finding of this thesis is that with the use of PI, results can be compiled as a template for predicting the structure-function state of pea plants at any plastochron age, under any growth conditions, before using small representative sample populations.

CHAPTER 1: General Introduction

Growth measurements such as relative growth rate (RGR), absolute growth rate, leaf area ratio (LAR) and leaf area index (LAI) involve destructive sampling techniques which prevent the same plant from being used for measurements at different stages in its development. These parameters can therefore only be used through periodic sampling of a population (Groot and Meicenheimer 2000a). Alternatively, the study of plant growth and development may be carried out using histological examination, chemical analysis, metabolic or molecular studies. However, there is a need to relate the collected data to time if they are to have meaning in developmental terms.

Accumulated growth data is also usually plotted against the plant's chronological age. However, chronological age e.g. using days after sowing (DAS), or days after germination (DAG), as a time variable is not the best choice because often inherent variability will become evident among plants sown at the same time. These can be large enough to obscure developmental trends (Groot and Meicenheimer 2000a). In other words, plants of the same chronological age may have reached different stages of development, while plants that are morphologically similar, may be of quite different chronological ages (Erickson and Michelini 1957; Lamoreaux *et al*. 1978).

Taking statistical precautions such as making use of large sample size, and randomization, may also result in significant relationships being obscured (Erickson and Michelini 1957). If the interest is in studying deterministic genetic factors in a developmental context, then experimental protocols must be devised which will compensate for the random factors that may influence the usefulness of data collected from a population of plants (Groot and Meicenheimer 2000a). Experimental variability can only be reduced when plants are not only genetically uniform but also maintained under same conditions of growth or through stringent control of the conditions under which experiments are conducted (Erickson and Michelini 1957; Lamoreaux *et al*. 1978).

1.1 The Plastochron index –a unit of developmental age

Erickson and Michelini (1957) are acknowledged for having developed the concept of using a numerical index to represent the developmental status of a plant; whereby observations were related to time directly by way of the numerical index. The purpose of the index was to minimize the difficulties inherent in developmental studies, which attempt to correlate morphological and physiological observations (Lamoreaux *et al* 1978). Simply put, Erickson and Michelini (1957) reasoned that if leaves were initiated at a uniform rate and if morphogenesis represented a continuum, then it should be possible to develop an index

for relating morphogenetic measurements to anatomical, physiological, and biochemical events or processes (Larson and Isebrands 1971).

Erickson and Michelini (1957)'s concept was derived from the term 'plastochron' which was first proposed by Askenasy (1880) as the interval of time between formation of two successive internode cells in *Nitella flexilis* (cited by Erickson and Michelini 1957). Erickson and Michelini (1957) selected leaf length as a measure of leaf development and extended Askenasy's definition to include the time interval between corresponding stages of development of two successive leaves. A plastochron might thus be more broadly defined as the interval between corresponding stages of development of successive leaves. The reference stage of development could be set at any determinate point: initiation, maturity, or any intermediate stage of development. According to Erickson and Michelini (1957), a plastochron can serve as the unit of developmental scale, when successive plastochrons are equal in duration.

Using *Xanthium*, a formula for plastochron index was derived as follows

$$PI = n + \frac{\log L_n - \log ?}{\log L_n - \log L_{n-1}} \qquad (1)$$

where,

n is the serial number (counting from the shoot base) of that leaf which just exceeds a certain length ? mm,

log L*n* is the natural logarithm of the length of the leaf *n* in mm,

log L*n+1* is the natural logarithm of the succeeding leaf whose length is less than ? mm

A plant is thus *n* plastochron old when the length of the leaf *n* is exactly ? mm. Erickson and Michelini (1957) showed that PI is linear over time. The inverse of the slope of the linear graph serves as the average duration of the plastochron. Leaf plastochron index (LPI) is calculated as

$$LPI_n \; ? \; PI \; ? \; n \qquad (2)$$

which is derived by simply subtracting the leaf's serial number from the plant's plastochron age (Erickson and Michelini 1957). The LPI represents the age of a leaf in plastochrons. A leaf of reference length ? mm is 0 plastochron old. The LPI is negative for leaves shorter than, and positive for leaves larger than, the reference length (Groot and Meicenheimer 2000a). The use of the plastochron index allows plant and leaf physiological age to be determined at any stage of development.

The plastochron index therefore provides a strict, mathematical basis for growth related processes, as it provides a morphological time scale, which has been shown to be more reliable than chronological age in studies in which morphological and physiological development of a whole plant, or plant organs is investigated (Lamoreaux *et al*. 1978).

Michelini (1958) proved this when he compared values for fresh weight, chlorophyll content and oxygen uptake in *Xanthium* plotted against time with plots of these three variables against LPI. A linear relationship was found between these variables and LPI while extreme variability existed when plotted directly against time.

Various morphological and physiological activities in plants have been related to a plant's and leaf's plastochron age. Lamoreaux *et al*. (1978) provided a comprehensive report of the works that had been carried out over two decades after Erickson and Michelini (1957) first developed the methodology. Leaf development has been described and correlated with plastochron age in *Xanthium* species (Maksymowych 1959; Maksymowych and Erickson 1960) and in Arabidopsis thaliana (Groot and Meicenheimer 2000a and 2000b), with results presented in terms of absolute and relative rates. In his monograph, Maksymowych (1973) reviewed how he and his associates had utilized PI and LPI to specify the developmental age of *Xanthium* plants with statistical precision. In another example, the fixation pattern of 14C within developing leaves of Eastern cottonwood has also been correlated to plastochron age by Larson *et al*. 1972 and the changes in the incorporation pattern of $^{14}CO_2$ into soluble proteins in *Xanthium* was related to LPI by Loewenberg (1970). Larson and Isebrands (1971) used the plastochron index as a quantitative determinant of a cottonwood tree developmental stage; and correlated dependent

variables such as leaf length, leaf area and leaf weight to LPI. Changes in temperature have been shown to cause changes in plastochron index in *Sorghum bicolor* (Quinby *et al*. 1973) and *Nicotiana tabacum* (Raper *et al*. 1975). Wimmers and Turgeon (1991) related sucrose movement in sink/source pea leaves to LPI; Gagnon and Beebe (1996a) correlated sink-to-source transition in cottonwood to LPI. Harney (2003) analyzed the effects of sulphur nutrition and light intensity on sulphur deficiency symptoms in *Phaseolus vulgaris*. From the above, it should be clear that many studies have been carried out using the plastochron index.

1.2 Using PI as a predictor.

Larson and Isebrands (1971) created models which, according to them, demonstrated that the PI and LPI could serve two useful purposes when applied to developmental studies of woody plants:

to adjust growth or other experimental parameters of plants of different developmental stages to a standardized morphological time scale, and

to predict developmental processes and events by means of simple, non-destructive measurements.

Larson and Isebrands (1971) argued that the main purpose of the use of PI is to assist with prediction of other correlations or biological events from relatively simple measurements. For example, if it can be demonstrated that the plastochron interval is constant for given growth conditions, then it may be assumed that other developmental processes

will also be proceeding at a uniform rate since they are linearly related to morphological time. Consequently, it should be theoretically possible to predict rather more quantitative data according to LPI, even though it is acknowledged that the prediction of quantitative data is subject to stringent limitations, it is argued that the prediction of qualitative biological events according to LPI can be both highly accurate and extremely useful (Larson and Isebrands 1971).

Larson and Isebrands (1971) showed that leaf maturity occurred at LPI 5 in 16-leaf plants of cottonwood and went on to correlate PI with the transition of the vascular tissue in the internode from the primary to the secondary state. Larson and Isebrands (1971) further concluded that through the use of LPI data, it is possible to select in advance, plants with a known stage of vascularization up to, and including the transition stage. These authors further stated that these correlations could be extended over time to plants grown under similar conditions but planted at different dates. In other words, the PI represented a real plant-related process.

Xanthium sp. is the most widely studied using the plastochron index formula given by Erickson and Michelini (1957). Plant processes such as leaf development (Maksymowych 1959; Maksymowych and Erickson 1960), petiole development and xylem differentiation (Maksymowych and Maksymowych 1982), incorporation of $^{14}CO_2$ into

soluble proteins (Loewenberg 1970), chloroplast growth (Holowinsky *et al*. 1965), changes in isoenzyme patterns (Chen *et al*. 1970), and flower initiation (Jacobs 1972) have been correlated with PI. It can thus be argued that compilation of these results can serve as a useful predictor of the morphological and physiological state of any *Xanthium* plant of known plastochron age, grown under similar conditions.

However, the use of plastochron index, fascinating and useful as it is, lost favour gradually and has become a rare experimental tool in recent times. More recent research carried out using PI has often been conducted based on authors' own discretionary reference length for calculating plastochron index in the same plant species. More often, the researchers do not rigorously adhere to, or test for, the assumptions of the PI (Harney 2003). Therefore, studies by different authors on the same plant species using the plastochron index, cannot be compiled in any sequence or meaningful way, as different formulae for PI have been used by these various authors. For example in *P. sativum*, PI has been calculated using internode length (Miller 1960), stipule length and width (Meicenheimer *et al*. 1983), leaf length (Wimmers and Turgeon 1991) and some researchers working with primodia tissues such as Gould and Cutter (1985) used an arbitrary plastochron value depending on the relative size of the leaf primodium. Clearly, an argument can be made for using PI but that thus requires the use of an accurate and functional PI formula; resulting in a constructive growth

model of the plant; which can serve as a predictor of the structure-function state of the plant. Only under these conditions, can the application of a uniform formula provide data which may be directly compared with other data, from experiments undertaken at a different time, location or varying conditions. This thesis explores several important physiological and structural processes using the model plant, *Pisum sativum*.

1.3 Why Peas?

Peas have been grown as important source of animal feed and human food for many centuries. Several thousand varieties exist throughout the world. Garden peas (*P. sativum* L.) are the fourth most important legume crop worldwide. They are used as good sources of protein in both food and feed (Cousin 1997). As such, considerable effort has been devoted to studies of the morphology, phenology, physiology and ecology of *P. sativum* under different environmental conditions. Aside from economical value, peas are good tools in laboratory experiments, as they have very short life span and produce leaves at a very fast rate, thus affording the opportunity of several leaf generations in a relatively short time. Peas are dwarf plants, offering an immediate advantage, as they can be grown in controlled environment cabinets throughout their life cycle. Peas are thus ideally suited to research in which the plastochron index forms the basis for determining plant or leaf age.

1.4 Past studies using the plastochron index on *P. sativum*.

As mentioned previously, the use of the plastochron index in the study of the growth of pea plants has been inconsistent in application and determination. This is because the pea leaf is awkward to measure, as each leaf axis is terminated by a tendril, and the developing leaf is in any case, tightly enclosed by the stipules during the early stages of leaf expansion (Erickson and Michelini 1957). According to Erickson and Michelini (1957), Higgins (1952) proposed a developmental index for phenological observations of cultivated peas, which had much in common with the plastochron index of Erickson and Michelini (1957). In this paper, Higgins (1952) proposed counting the number of "fully-developed nodes" on a pea plant, and adding to the integer, a decimal fraction purportedly representing the stage of development that had been reached by the "terminal bud". The integral part corresponded to the integral part of the PI of Erickson and Michelini (1957). However the authors reported that Higgins' index is not clearly linear with time for a fractional part of a plastochron. From preliminary observations, Erickson and Michelini (1957) concluded that it seemed more appropriate to use internode lengths in calculation of a plastochron index in peas. They suggested that each internode should be assigned the same serial number as that of the leaf subtending it, and using internode lengths in formula (1) in place of leaf lengths. Furthermore, the reference internode length should be taken as 20mm.

Several studies were subsequently undertaken using *P. sativum* using the plastochron index. For example, the correlation of leaf plastochron age with Hill-reaction activity of chloroplasts (Miller 1960); meristem characteristics of genetically modified pea leaf primodia (Meicenheimer *et al*. 1983); changes in volume and cell number in the different regions of the shoot apex (Lyndon 1968); morphogenesis of the compound leaf in three genotypes of the pea (Gould and Cutter 1985); appearance of transfer cells and solute uptake in minor veins of *P. sativum* leaves (Wimmers and Turgeon 1991); pea leaf, leaflet and tendril determination from primodia (Gould *et al*. 1994). Most of these studies were carried out using other parameters (based on authors' discretion) than internode length and values other than 20 mm. Thus, the results determined by various authors cannot be correlated like those of *Xanthium* because of the difference in base parameters which were used to calculate the plastochron values in each case. It is quite clear from the above, that a plant or leaf stated as being of, or at a certain plastochron age in one study, will not be calculated or reflected as being same plastochron age in another study.

1.5 Research objectives

The first task in this thesis was to explore the various parameters that have been used in calculating plastochron index in the past and to choose a more suitable parameter in determining plastochron index in peas. The formula derived as a result could then be used to carry out

various morphological and physiological studies that when put together, hopefully, would serve as a template for the prediction of the growth, development and reactions of *Pisum sativum* L. to different growth conditions.

My research would therefore involve the use of the developed PI formula to conduct key quantitative and qualitative investigations, in which all data and observations are related to PI and LPI. The research will endeavour to investigate two main hypotheses in validating the authenticity of the plastochron index as a growth scale:

If plastochron index is a useful tool in assessing plant growth, then both qualitative and quantitative plant growth and developmental states should be related to PI based on a single PI formula.

If PI is a morphological time scale, then plants at the same plastochron index should be at similar developmental state, irrespective of the growth condition.

1.5.1 Qualitative analysis using PI as time scale

Qualitative analysis could be useful in investigating the two hypotheses surrounding PI: it could not only reveal the relationship between structure-function changes in tissues and PI; but it could also indicate if plants at the same LPI under different growth conditions, were at a different structure-function state.

1.5.1.1 PI in physiological studies: A case study of the sink to source transition of P. sativum leaves via phloem transport transition:

The passage from an entirely heterotrophic, carbohydrate-importing organ to an entirely autotrophic, carbohydrate-exporting organ is known as the sink-to-source transition (Turgeon 1989; Robinson-Beers *et al*. 1990; Gagnon and Beebe 1996a). 'Matured' leaves are carbon sources, while the 'immature' leaves are carbon sinks. The former, exports while the latter, imports assimilate. The leaves at the transition state are 'maturing' leaves, which import and export assimilates at the same time. However, the question is, 'when has a pea leaf matured? It has been reported that leaves of dicotyledonous plants stop importing and begin to export when they are 30-60% fully expanded (Turgeon 1989). In dicotyledonous plants, the transition from photoassimilate sink to source status begins shortly after the leaf has begun to unfold. At this point in development, the major morphogenetic events that determine leaf shape are considered to be over (Geiger 1979; Turgeon 1989; Minchin *et al*. 1993).

Gagnon and Beebe (1996a) have previously shown that the number of leaves under sink-to-source transition state can be predicted using PI value. Wimmers and Turgeon (1991) had earlier used leaf length in calculating PI in peas and proceeded to relate sink-source transition in peas to LPI. With the use of [14C] sucrose, they reported that sink-

source transition occurs at LPI 1.6 -1.8 in the dwarf-pea plant (*P. sativum* L. cv. Little Marvel). It was therefore envisaged that, my research would include the use of the PI formula to study the developmental and functional changes in phloem transport pathway in relation to leaf development vis-à-vis the leaf plastochron index. The principal interest would be expressing the results obtained based on the sink/source pattern of transport between attached compound leaves and within paired leaflets.

1.5.1.2 PI in morphological studies: a case study of the sink to source transition of P. sativum leaves using anatomical transition:

Structural and physiological changes associated with sink-source transition are initiated at the tip of the expanding leaf and progress basipetally. As such, a maturation gradient exists that can be exploited experimentally (Turgeon 1989). The differences in phloem ultrastructure between source, sink and transition regions indicate that the maturity of the vascular system is in some way related to the import or export status (Fellows and Geiger 1974). Most of the photoassimilates imported by sink leaves is unloaded by moderately large veins and the smallest veins are thought to be relatively, or completely unimportant in this regard. Turgeon (1989) argued that since the tissue is compact during the import phase, the finest minor veins are possibly not needed for distribution of the imported assimilate; therefore leaves at this stage of development will still be

expanding and the minor-vein phloem must still retain the capacity for further elongation.

In this part of the thesis (chapter 4) I report on my investigation of sink to source transition, and include evidence from an anatomical investigation of leaf development in relation to leaf plastochron index, using light and transmission electron microscopy.

1.5.2 Quantitative analysis using PI

Growth rate in studies involving the plastochron index is simply expressed as the change in PI over time (Erickson and Michelini 1957). Plastochron index (PI) is not only used in determining plant and leaf morphological ages; but also the periodic leaf length measurements used in plastochron index analysis can be used in determining leaf elongation rate, while plastochron duration is invariably the rate of leaf appearance (Ade-Ademilua and Botha 2005). It was hoped that by using PI, different forms of accessing plant growth rate can be explored and the growth of a particular plant and leaf can be monitored over time faster and accurately.

1.5.2.1 PI in growth rate studies: a case study of the effects of elevated CO_2 and nitrogen availability on plant growth rate.

The interactive effects of elevated CO_2 and other environmental factors on plants remains a subject of much discussion. Though some workers have proposed that high [CO_2] effects on plant growth were not

affected by environmental stress factors (Idso and Idso 1994), others have reported or concluded that high [CO_2] effects vary among plant species grown under different environmental conditions (Kimball 1983, Poorter 1993, 1998, Thompson and Woodward 1994, Hunt *et al*. 1995, Ziska *et al*. 1996, Brunce 1998, Wu and Wang 2000). According to Kramer (1981) and Poorter (1998), the positive effects of CO_2 cannot be mentioned or inferred when other environmental factors are limiting. In the words of van der Galiën (2002), 'the agriculture might be affected because many of the carbon dioxide damage sensitive plants are nutritious crops, essential for the food production, especially in the Third World. The industrialized world can afford to genetically engineer these plants and bring them to even higher yields; but will the poor Third World be able to buy the expensive seeds?' Knowledge of the interactive effects of CO_2 with environmental factors (such as temperature, light, and nutrient availability) on the growth of crops would enable us to predict not only the future of food availability in Africa, but perhaps changes in nutritional quality. The research will therefore include the use of PI in analyzing the growth of *P. sativum* L under different CO_2 and nitrogen conditions.

According to Reddy *et al.* (1998), the effects of increased [CO_2] on growth is primarily expressed in changes in leaf area production with smaller effects of increased CO_2 on photosynthetic rate, nitrogen and water use efficiency. Under elevated CO_2, leaf area responses may be

limited by deficits of nutrients or water (Reddy *et al*. 1998). Plastochron index will therefore be a vital tool in analyzing leaf appearance and expansion/elongation rates which according to Reddy *et al*. (1993), are factors involved in determining leaf area changes under different growth conditions.

Many of the reports on the effects of growth conditions such as high [CO_2] and [N] on plant growth are based on the comparison of tissues of same chronological age or size. With the use of both qualitative and quantitative analyses, the research upon which the thesis is based seeks to explore the fundamental question:

Are plants/leaves of the same chronological age under different growth conditions, at the same PI value and vice versa?

I believe that some differences in growth and development of plants under different growth conditions reported by some workers are actually a function of differences in plastochron index of plants compared. I will therefore attempt to show that differences in growth and development under different growth conditions can be explained in part at least, to be due to differences in plastochron index. This thesis demonstrates the importance of taking a quantitative measure of plant age, as plant age affects and effects many key changes in the growth of plants. This study using the pea plant demonstrates that PI is a powerful tool which can be used to determine key growth parameters.

CHAPETR 2: Materials and Methods

2.1 Plant Culture

Sterilized (3.5% m/v sodium hypochlorite solution to prevent nodulation; Rivière *et al.* 1996) and non-sterilized seeds of *P. sativum* var. Greenfeast were sown in sterile and non-sterile potting soil respectively (Greenfingers, South-Africa). Six seedlings were transplanted per pot. Five g of slow-releasing fertilizer (NPK 2:3:2; Wonder Horticultural Products, Johannesburg, South Africa) was added to the soil in pots (185 x 185mm, 165mm deep) prior to transplanting seedlings. In all, twelve plants were used per treatment. Pots were irrigated with either full strength complete (with 6mM nitrogen) or nitrogen-free Long Ashton nutrient solution (Hewitt 1966) depending on treatment group

2.2 Plant growth parameters

Treatments were based on six controlled parameters: elevated CO_2 (E, 1000 µmol mol^{-1}) and ambient CO_2 (A, 380 µmol mol^{-1}); non-nodulating (r) and nodulating (R); irrigation with nutrient solution containing mineral N (N) and irrigation with nitrogen-free nutrient solution (n). Pots were assigned symbols as described in the table below:

ABBREVIATION	DENOTATION
rAN	**Non-nodulating/non-nitrogen fixing plants supplied with N+ nutrient solution under ambient CO_2.**
rEN	**Non-nodulating/non-nitrogen fixing plants supplied with N+ nutrient solution under elevated CO_2.**
RAn	**Nodulating/ nitrogen fixing plants supplied with N-free nutrient solution under ambient CO_2.**
REn	**Nodulating/ nitrogen fixing plants supplied with N-free nutrient solution under elevated CO_2.**
RAN	**Nodulating/ nitrogen fixing plants supplied with N+ nutrient solution under ambient CO_2.**
REN	**Nodulating/ nitrogen fixing plants supplied with N+ nutrient solution under elevated CO_2.**

The group, non-nodulating supplied with N-free nutrients solution were left out of the experiments because preliminary tests under both controlled and greenhouse conditions show that without nitrogen supply, non-nodulating pea seedlings display highly retarded growth and plants die within a few weeks of seedling germination; which finds support in the work of Arnone and Gordon (1990), who also demonstrated that in un-inoculated red alder, plants receiving nutrient solution without nitrogen do not grow. Pots were placed in

randomised blocked matrix design and positions were changed in a definite pattern daily to prevent any accumulative chamber position effect.

2.3 Growth Conditions

Plants were grown in two growth chambers (Conviron Model S10H, Controlled Environments Ltd, Winnipeg, Canada) maintained under 25/18°C day/night regime, 68-70 % Relative Humidity and with a 16-h photoperiod. Ambient and elevated CO_2 concentrations were maintained at 380 and 1000 µmol mol^{-1} respectively with insignificant fluctuations within ±15 µmol mol^{-1} of the [CO_2] set point. CO_2 was monitored integrated computer-controlled Horiba APBA-250 indoor CO_2 monitor (Horiba Ltd, Japan). Plants were illuminated using a combination of fluorescent tubes (F48T12.CW/VHO1500, Sylvania, USA) and frosted incandescent 60W bulbs (Philips, Eindhoven, The Netherlands). Photosynthetic active radiation (PAR at 400 – 700 nm) was set, such that it was about 250 µmol m^{-2} s^{-1} when measured 20 cm above soil level (as recommended by Olivier and Annandale 1998), with a Li-85A Quantum sensor (Li-Cor Inc, Nebraska, USA). Pots' positions were changed every day along a matrix pattern, to avoid chamber effect.

2.4 Leaflet measurements

The first true leaf of the pea plant (with oval shaped leaflets and tendrils) is borne on node 3. Node numbering is taken from the base starting with zero. Leaflet length measurements were recorded from the first pair of leaflets on node 5. Measurements were made using an electronic digital calliper at the same time each day, throughout the vegetative growth stage.

2.5 Leaf clearing

The clearing technique described by Shobe and Lersten (1967) was adopted in clearing pea leaflets at different plastochron ages. Due to the fragile nature of the leaflets, they were soaked in 10% aqueous sodium hydroxide (NaOH) solution at room temperature for 3-4 days. The NaOH solution was changed repeatedly until leaflets cleared. The cleared leaflets were rinsed in distilled water for 15 min. Leaves were then soaked in saturated aqueous chloral hydrate at room temperature for 2-3 days. The lamina became opaque, whilst veins remained white.

2.5.1 Staining and mounting of cleared leaves

After clearing, leaflets were gently rinsed in distilled water, then dehydrated with 30, 50, 70, 90 and 100% ethanol for 5 minutes each at room temperature. Cleared leaflets were then placed in safranin O in

100% ethanol for staining for 24 h. Destaining was done by rinsing leaflets in 100% ethanol and then halted using 50:50 (v : v) ethanol : xylene solution. Leaflets were soaked in xylene for 3 days to remove every trace of ethanol or water. Each leaflet was then mounted between glass slide and cover slip with Canada Balsam and hardened in oven at 45 ºC for 2 weeks. Slides were observed under bright field using the Olympus BX61 microscope. Montage images were constructed of the whole leaf using 2x objectives.

2.6 Plastochron analysis

The average leaflet lengths were recorded for each node on the plants from 11 DAG to 20 DAG since plastochron analysis is limited to period of active vegetative growth. Plastochron age of plants (PI) per day was calculated for each plant and leaf plastochron age (LPI; See chapter 1 for formula). The leaf plastochron age was determined using leaflets on node 6 from the day they were unfolded, till when they stop elongating.

2.7 Surface loading of phloem transport tracer

All experiments were carried out using intact plants. The leaf loading protocol used to load 5,6-CF was adapted from that of Grignon *et al*. (1989) and Roberts *et al*. (1997). Experiments were carried out by applying 5,6-CFDA to source leaflets and monitoring the transport of the fluorophore in leaflets above the fed leaflet. Another set of

experiments was carried using leaflets of all LPI groups, in which the transport of the fluorophore into the opposite leaflet (paired to the fed leaflet) and petiole between the paired leaflets was monitored. Experiments involving the application of 5,6-CFDA were undertaken 7 h after the beginning of the photoperiod. In all cases, the adaxial surface of an attached leaflet was gently abraded with fine sandpaper, and rinsed with distilled water,. Lamina zones lacking large veins were chosen as abrasion sites. After abrasion, 100 µL of working strength 5,6-carboxyfluorescein diacetate (5,6-CFDA, see 2.8 below) was applied directly to the abraded area to the leaflet surface and covered with transparent polythene film (Housebrand, Brackenfell, South Africa) to prevent evaporation. At the end of the leaf loading experiments, the leaflets and petioles of interest, were detached 30 min to 3 h after application of 5,6-CFDA and observed for 5,6-CF distribution, using an Olympus BX-61 (Tokyo, Japan) epifluorescence microscope fitted with a U-YFP filter set (10C/Topaz 41028, Chroma Technologies, Battlebro USA). Several montages of all regions of each leaflet were taken using an Olympus PlanApo 2x objective (Tokyo, Japan) and the whole leaflet images were reconstructed using the multiple image alignment (MIA) routine in analSiS 3.2 (Soft Imaging System GmbH, Münster, Germany).

2.7 Effects of illumination

Experiments were also undertaken by feeding 5,6-CFDA to a predarkened leaflet, and the resultant movement of the cleavage product, 5,6-CF across the internode into the illuminated opposite leaflet of the pair was examined 30 min to 3 h after application of the fluorophore. Conversely, the illuminated leaflet was offered 5,6-CFDA and the transport of the cleavage product, 5,6-CF into the darkened leaflet was studied. Experiments were repeated six to eight times and the micrographs are thus representative of the treatment regimen described above.

2.8 Preparation of working strength 5,6-CFDA

5,6-CFDA (C-195) was purchased from Molecular Probes, Eugen, Oregon USA in 100 mg units, to which 1 ml of 0.2% dimethylsulphuroxide (DMSO) was added. This stock solution was foil-wrapped and stored at -4 ºC until needed. Working strength 5,6-CFDA was prepared by taking a 1 µL aliquot of the stock solution and adding to 1 mL of distilled water. Working strength 5,6-CFDA was foil-wrapped and stored at -4 ºC until needed. This resultant mixture was applied directly to abraded leaflet surfaces. Once loaded the acetate moiety is cleaved and the resultant 5,6-CF is transported within the phloem.

2.9 Embedding tissues for light microscopy

The technique published by Feder and O'Brien (1968), adapted for paraffin wax embedment was used in all cases to prepare sections for light microscopy. The petiole between paired leaflets was excised and fixed in 10% acrolein at 0ºC for 24 h. Tissue samples were dehydrated with 2-methoxyethanol (synonyms: ethylene glycol monomethyl ether; methyl cellosolve) at about 0ºC with a change in the solvent in 24 h. Further dehydration was carried at 0ºC for 24 h per solvent in 100% ethanol, n-propanol and finally n-butanol respectively, followed by infiltration and embedment in paraplast wax by transferring tissues from n-butanol to a mixture of n-butanol and paraplast wax chips (1:1 v:v) at room temperature and then subsequently transferred to an oven (Labcon (PTY) Ltd., Krugersdorp, South Africa) at 60ºC for 24h. After which, three changes in 100% liquid paraplast wax at 60 ºC at 12 h interval were carried out, followed before blocking the tissue and sectioning at 12 µm using a Leitz Wetzlar Minot rotary microtome (Wetzlar, Germany). Serial sections were mounted on slides with adhesive, dried in the oven at 45 ºC, dehydrated and processed for staining in safranin, crystal violet and Fast Green FCF (0.5% in equal parts of methyl cellosolve, absolute ethyl alcohol and clove oil) before fixing permanently using Canada balsam. After which the slides were dried at 45 ºC for 2 wks. Fixed sections were observed bright field using Olympus BX61.

2.10 Transmission electron microscopy

Tissues (1x2 mm) from the upper and mid-section of the lamina of leaflets at different plastochron ages with minor veins perpendicular to the longer side of the section, were cut and fixed in a cold solution of 2.5% glutaraldehyde and 0.5% paraformaldehyde in 0.2M sodium cacodylate buffer for 24 h at 4 ºC. Buoyant tissues were evacuated by placing vials with fixative in a vacuum dessicator and gradually pulling a slight vacuum (16.93 KPa). Vials were left under vacuum for about 1 h and then returned to the refrigerator. Tissues were then rinsed for 30 min with two changes in 0.2M sodium cacodylate buffer and the post-fixed in 2% osmium tetroxide solution at 4 ºC for 4 h, after which tissues were rinsed for 30 min with two changes in cacodylate buffer. Fixed tissues were the dehydrated through a series of 30 (5 min), 50 (5 min) and 70% (overnight) ethanol at 4 ºC and then further dehydration were carried out at room temperature with 80 (5 min), 90 (5 min) and 100 % (10 min with two changes) ethanol, and further 30 min with two changes in propylene oxide. Plastic infiltration of the tissue was carried out by putting tissues in series of 75:25 (3 h), 50:50 (overnight), 25:75 (6 h) v:v propylene oxide : ERL-SPURR epoxy resin (Spurr, 1969). Tissues were then left in pure resin overnight. Tissues were then placed in fresh resin in capsules for polymerisation at 60 ºC for 36 h. Ultrathin (gold-silver) sections were cut using a diamond knife mounted on a RMC MT-7 ultra microtome (Research & Manufacturing Co. Inc., Tucson, Arizona). Sections were collected on

Formvar©-coated single-slot copper grids and stained for 30 min in uranyl acetate (2% in H_2O) and 5 min in Reynolds lead citrate (Reynolds, 1963). Sections were viewed and photographed using a Jeol JSM 1210 transmission electron microscope (Tokyo, Japan).

2.11 Statistical analysis

Leaflet, stipule and internode lengths were recorded for each node. Data were analyzed for each plant. Twelve plants were used for each analysis. Descriptive and regression analyses were carried out using Excel 2000. One-way analysis of variance (ANOVA) was carried out at 0.1, 1 and 5% levels of significance. Unless otherwise stated, experiments were repeated at least four times.

CHAPTER 3: Defining Growth and Plant Age by Determination of the Plastochron Index in *Pisum Sativum* L.

3.1 Introduction

Plant growth is a complex process, involving cell division differentiation and morphogenesis. These overlapping processes result in the formation of a plant body, which, as it grows, will contain regions in which cells and tissues as well as organ subtended by this structure, are at various stages of growth. Determination of growth, growth arte, relative growth rate amongst others, posses several problems therefore, due specifically to the gradual change in the state of differentiation of organs or a plant.

In efforts to overcome the inherent problems of determining growth, researchers have used age in days or weeks after emergence of an organ, or days after germination, routinely to express the "age" of a plant. However, what is age? This is the principal question which is addressed by this Chapter, in which I explore the use of the plastochron index as a reliable measure of plant age, and demonstrate the relationship between organ (leaf) initiation, growth and state of maturity.

According to Erickson and Michelini (1957), a plastochron is broadly defined as the interval between corresponding stages of development

of an organ in succession. The organ used by Erickson and Michelini (1957) to derive the formula for plastochron index (PI) in *Xanthium*, and thereafter used by other authors on most plants, is the leaf.

However, as discussed in Chapter 1 the pea leaf is awkward to measure. Each leaf axis is terminated by tendril(s) and the developing leaf is tightly enclosed by the stipules during the early stages of leaf expansion. As a result, Erickson and Michelini (1957) proposed the use of internode length in the calculation of the PI in peas, rather than the more conventional unit of leaf length. Thus for peas, Erickson and Michelini (1957) replaced leaf length with internode length in equation 1 (Chapter 1), each internode is assigned the same serial number as that of the leaf subtending it, and the reference internode length was set at 20mm. Erickson and Michelini (1957) formula for determining plastochron index (PI) in peas is thus

$$PI = n - \frac{\log L_n - \log 20}{\log L_n - \log L_{n-1}} \qquad (3)$$

where *n* is the serial number (counting from the shoot base) of the leaf subtending that internode which just exceeds 20 mm;

log L*n* is the natural logarithm of the length of the internode *n*, and

log L*n+1* is the natural logarithm of the length of the succeeding internode with a length that is less than 20 mm.

Miller (1960) subsequently used this formula in his study involving the correlation between leaf plastochron age and Hill-reaction activity of

chloroplasts in peas. As highlighted in Chapter 1, studies involving the use of plastochron age in *P. sativum* have however, taken different forms from the use of internode length: Meicenheimer *et al*. (1983) used stipule length and width to calculate stipule age. Their approach is however, vague and not too clear. Lyndon (1968) defined the nth leaf primodium produced as being *n* plastochron old, and each had according to the authors nine morphologically recognizable stages of primodium development defining their 0.1 plastochron units. Meicenheimer *et al*. (1983) determined shoot age by using the measured radii from the central protoxylem elements, or procambium of each leaf primodium to the center of the apical meristem. Gould and Cutter (1985) defined the plastochron age of a leaf primodium as the number of visible leaf primodia initiated on the shoot meristem after its own initiation plus one. Gould and Cutter (1985) gave a leaf primodium an arbitrary plastochron value depending on its relative size. Wimmers and Turgeon (1991) used leaf length in calculating PI in their study of the transfer cells and solute uptake in the dwarf-pea plant (*P. sativum* L. cv. Little Marvel). However, they did not state if leaf measurement did or did not include the apical tendril.

Clearly, all of the methods outlined above are either not explicit, quite complicated or involve damaging the plant in some way. In contrast, PI calculations based on Erickson and Michelini's (1957) formula can be determined for leaf primodia which are inaccessible, without

dissection; as well as for older leaves which are no longer growing exponentially, or even for those which have stopped growing entirely (Erickson 1976). Preliminary experiments undertaken in this study suggested that using internode length in determining PI in tropical pea varieties with a reference length of 20mm as suggested by Erickson and Michelini (1957) was not suitable for all varieties including the Greenfeast variety to be used in this research study.

The main purpose of this PhD research was to determine the use of PI as a tool in experimental analysis, and also to present data from different analytical areas that can be linked to each other as they are all connected with plant growth. A uniform PI formula was therefore essential for all analyses that were to be carried out. A chief concern was that it was essential to be able to calculate PI without damaging the plant (Erickson and Michelini 1957), and as such the plant organ used to calculate PI would have to meet the following criteria stated by Erickson and Michelini (1957), and Lamoreaux *et al*. (1978.

Early growth of the organ must occur at an exponential rate;

Early growth of successors on a single plant must occur at the same relative rate;

Successive plastochrons must be of the same duration for a particular plant.

Internode length, leaf length and stipule length are all parameters that have been used by various authors (see above) to determine plastochron index in *P. sativum* L. In using the internode length, the internode subtending a leaf at a particular node is measured in place of that leaf; leaf length involves measuring the leaf from the petiole axis to the tip of the tendril while stipule length is taken as the average length of the stipules at the base of the leaf. Leaflets are the only organs associated with the leaf that have not been used to determine plastochron index, yet the leaflet is the only organ that is most similar to the leaf on most dicotyledonous plants. Aside from Erickson and Michelini (1957) who pointed out that they decided on using internode length as against leaf length used by Higgins (as discussed above), other researchers did not state their reason for opting for stipule length or even the leaf length that has been condemned by Erickson and Michelini (1957). This Chapter is therefore a report of the experiments conducted in order to explore the organ that best fulfils the criteria highlighted above.

3.2 Results and Discussion

3.2.1 Internode length:

The internode lengths of plants 15 days after germination (DAG) are shown in Table 3.1. Table 3.1 shows that there is considerable variation in number of nodes produced as well as in the internode length in Greenfeast pea plants, even when grown under controlled

environmental conditions. Using the reference length (20mm) suggested by Erickson and Michelini (1957), then only plant 3 and 7 (data in bold typeface, Table 3.1) could be used to calculate plastochron age, and it is unrealistic (and misleading) to state that these plants were 3.48 and 3.23 plastochrons old by 15 DAG. Furthermore, neither will reach the 4th plastochron for an indeterminate time period.

An alternative option which was considered was to use a different reference length. It is important to note that, two criteria need to be satisfied before simply choosing a new reference length:

1. The reference length must be such that the length of internode *n* is equal or longer than the reference length, while length of internode *n*+1 must be shorter than the reference length.
2. All internodes developed before (i.e. older than) internode *n* must be longer than the reference length.

Fulfilling both criteria in all of the plants sampled and whose data are represented in Table 3.1 proved to be impossible. No matter what reference length was chosen, internode *n* either does not have a succeeding internode *n*+1 which is shorter than *n* (to fulfill criterion 1), or there are internodes preceding *n*, which are actually shorter than the chosen reference length. Criterion 2 could not therefore be met using internode length. Given that plants were grown under controlled environment, the variability of internode length precludes this as a reliable measure of age.

Table 3.1: Internode length (mm) of 10 P. sativum var. Greenfeast plants grown under controlled environment at 15 DAG.
Only data of plants in bold can be used to calculate plastochron index according to Erickson and Michelini's(1957) formula.

	Node 3	Node 4	Node 5	Node 6	Node 7	Node 8
Plant 1	16	18	12			
Plant 2	18	18	13	13	15	15
Plant 3	**22**	**18**	**17**	**17**	**8**	
Plant 4	16	16	15	19	14	
Plant 5	18	16	15	16	14	
Plant 6	18	16	16	15	12	
Plant 7	**21**	**17**	**17**	**17**	**15**	
Plant 8	18	13	13	15	12	
Plant 9	18	16	13	13	10	
Plant 10	17	14	15	14	14	4

3.2.2 Stipule length:

The proposal by Meicenheimer *et al.* (1983) to use stipule length was examined. Using stipule length measurement proved to be equally difficult. The stipules in peas sometime fold either inwards or outwards, thereby requiring manual unfolding that despite the outmost care, almost always resulted in injury and therefore in a reduction in the rate of elongation of the stipule after manipulation for measurement. The sides of the serrated base of the stipules also tend to cling to each other around the node, which made it difficult to determine the full length of the stipule without causing substantial injury to the basal cells of the stipule.

3.2.3 Leaf length:

The compound leaf of the pea is composed of a pair of stipules, several pair(s) of leaflets and terminates with one or more tendril(s). The length of the whole leaf is difficult to measure due to the curling of the tendril. Uncurling the tendril during measurement would, as with stipules, lead to tendril damage, which would effectively retard or prevent future elongation of the leaf that is being measured. Perhaps more important, is the fact that tendril growth is not time-, but proximity-based, and the growth of tendrils is thus highly dependent on the proximity of the tendril to a supporting object or structure. In other words, its length depends on the distance from the nearest support structure and elongation growth ceases in all cases as soon as

the tendril establishes good contact with its support. It would therefore be unrealistic to relate whole leaf (including tendril) elongation in peas to a time base for age, as one would have to ensure uniform, support distance conditions. Data from measurements reflected this with no apparent relationships.

3.2.4 Leaflet length:

Pea leaflets are analogous to leaves in most dicotyledon species and it therefore seemed logical to rather use leaflet length to determine plastochron age. Measuring leaflet length proved to be easier to accomplish as leaflets could be gently manipulated into a position to allow for, and to accommodate measurement without damaging them. As Erickson (1976) defined the PI in decussate-leaved plants as "the interval between initiations of successive pairs of leaves", it seemed logical to explore the plastochron in *P. sativum*, after defining it as the interval between initiations of successive first pairs of leaflets.

The appropriate reference length for estimating PI was determined in a series of experiments under controlled growth conditions. Leaflet length measurements were taken for a week, on a daily basis from 11 DAG. Mean leaflet length was plotted against time for each plant which shows the progressive sequence of leaflet development (Fig. 3.1). Leaflets show a typical growth and enlargement pattern, before transitioning from a log to a lag growth phase. The R2 values revealed

an almost perfect linearity during the log growth phase and the slope of the elongation rate of leaflets (in ten replicate plants) on nodes 6-10 of 10 plants sampled averaged 3.26 ? 0.29 with no significant difference at p=0.05. This indicated that early leaflet growth occurred at the same relative rate, irrespective of position. A leaflet on leaf *n* is about 20 mm long by the time the following leaf *n*+1 unfolds (highlighted in Fig. 3.1 by vertical dashed lines); therefore, a reference length of 20 mm seemed to be a realistic value on which to base the calculation of PI. The adapted formula for PI for *P. sativum* was therefore similar to equation (3) except that L*n*, L*n*+1, and the reference length 20 mm refer to average leaflet-length instead of the internode length as indicated in equation (3).

PI was calculated for each plant per unit time from 11 DAG, and PI vs. time was plotted for each individual plant. Figure 3.2 shows two examples in which variation in the rate of change of PI vs. DAG were noted with resultant changes in regression values. Figure 3.2 further shows that the relationship between the PI and time was linear for plant 1, but that this was not completely so for plant 2. These data find strong support in Erickson and Michelini (1957) results, as these authors too, reported similar variability using *Xanthium*. After about PI=11, a decrease in the slope of the curve was noted in some pea plants. The authors observed a similar decline in the slope of the curve for *Xanthium* by about PI=13, and suggested root binding of the plant

as a probable cause. The plants used in this study were also cultivated in pots, which could also conceivably have imposed limitations on root growth.

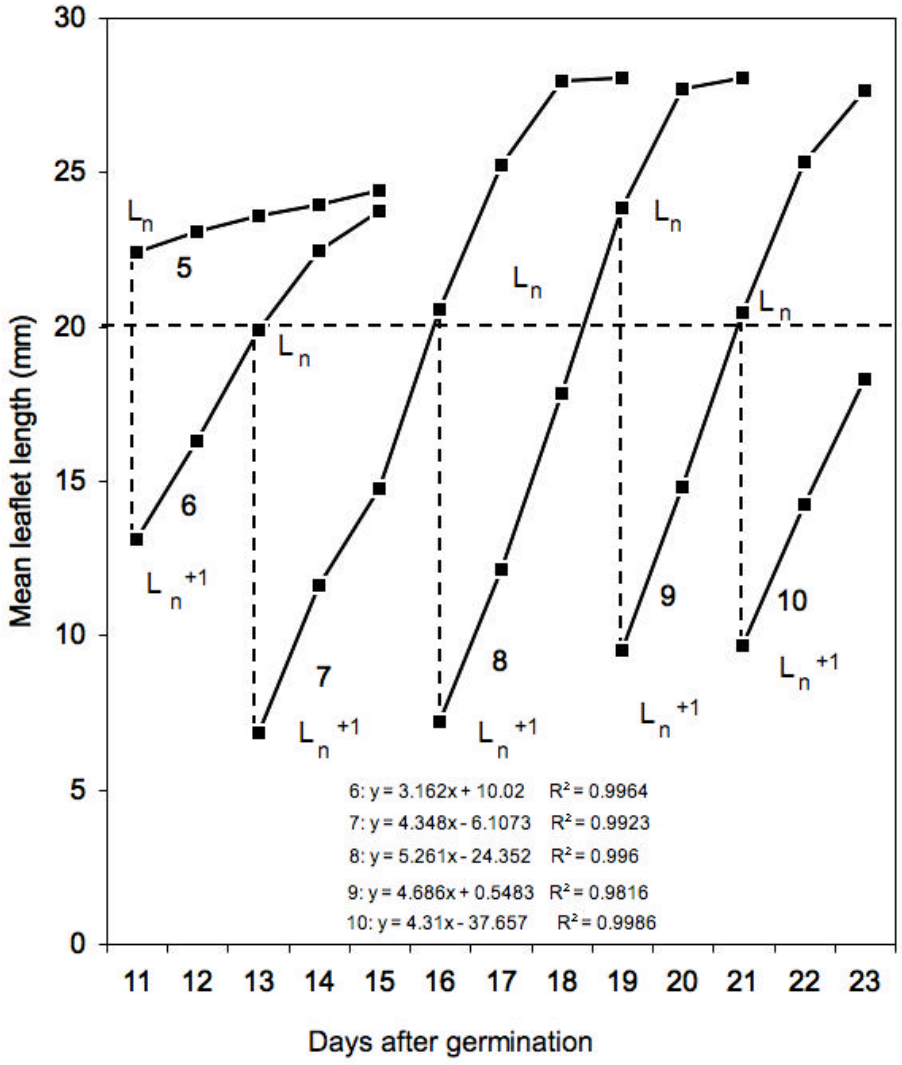

Fig. 3.1: Mean lengths of successive pair of leaflets of a P. sativum L. plant plotted against time. Each growth curve applies to leaflets on a particular node (node number indicated beside curve). The equations for the period of exponential growth for each line with R2 values are included. Vertical dashed lines indicate values needed to calculate plastochron index per time.

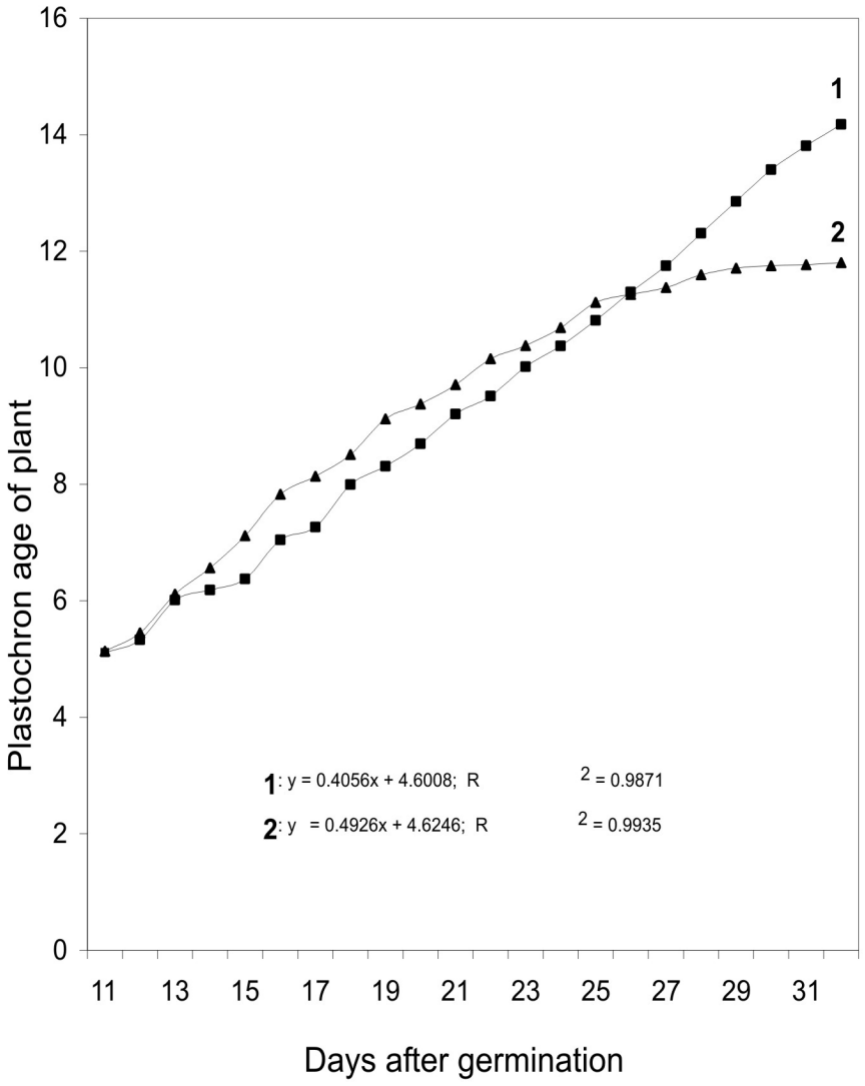

Fig. 3.2: Illustrates the rate of change in the plastochron index of two P. sativum var. Greenfeast plants (1 and 2). Embedded are equations for the period of exponential growth (11-20 DAG, about PI=11) with the R2 values.

Regression lines were fitted by least squares up to about PI=11 for all plants, as was the case in the data reported by Erickson and Michelini (1957). The SE of regression lines fitted for both plants in Fig. 3.2 was calculated to be approximately 0.01 while the slopes of the fitted lines were 0.42 and 0.43 day^{-1}. The average duration of the plastochron is invariably the reciprocal plot of the slope of a PI over time, which for the two plants illustrated in Fig. 3.2 are 2.38 and 2.33 days respectively. The standard errors for both plants were calculated to be approximately 0.01 plastochron, which represents approximately 0.02days. Extrapolating further, the standard error of PI is less than one hour! The highest standard error calculated for any of the other pea plants was 2 h (data is not shown). The data therefore compare favourably with Erickson and Michelini's (1957) where an SE of up to 7.63 h was recorded for the *Xanthium* plants sampled by these authors.

It is therefore clear that the mean length of successive pair of leaflets in determining plastochron age of *P. sativum* satisfies all the criteria listed below, in which leaf length is used for the determination of plastochron age:

Early leaflet growth occurs at an exponential rate – The data presented in Fig. 3.1 clearly shows that the data satisfies this criterion, with the mean length of successive pairs of leaflets increasing linearly (as shown by the R^2 values) with time during the early stage of the leaflet growth.

Early growth of successive leaves on a single plant occurs at the same relative rate - the graph for the successive pairs of leaflets (Fig. 3.1) have similar slopes and occur at approximately the same periodicity. Successive plastochrons are of the same duration for a particular plant - The statistical analysis of the data shows the average duration of a plastochron in *P. sativum* L. differs only in a few hours, which is less than that achieved by Erickson and Michelini (1957). Erickson and Michelin (1957) stressed that the PI serves to quantify the developmental status of a shoot with an accuracy of equal to, or less than a few hours.

In conclusion, the data accumulated from controlled environment-grown *P. sativum* L. plants shows that the formula for PI was indeed robust. As such Equation (2) would serve as the basis for the calculation and determination of LPI and PI. All subsequent experiments utilized Equation (2) as it was shown to be an accurate predictor of leaf age.

CHAPTER 4: Sink to Source Transition of *Pisum sativum* leaves in relation to Leaf Plastochron Index

Preamble

In this chapter the results of an in-depth investigation of the sink-to-source transition in compound leaves of *P. sativum* are reported. The study was conducted using experiments which illustrated the transport of 5,6-carboxyfluorescein (5,6-CF) from fed leaflets of known plastochron age to others in the sink-source transition. During the experiments, it became evident that phloem transport within the sink-source transition continuum did not simply highlight transport along the physiological gradient from source to sink, as expected. Two distinct topics are discussed under the broad heading sink-source transition. One deals with the process of 'Sink to source transition of *P. sativum* leaves in relation to leaf plastochron index' and makes use of the phloem-mobile fluorophore 5,6-CF to examine phloem transport between the compound leaves, and relates to sink-to-source transition to leaf plastochron index. The second aspect deals with a phenomenon that was seen in each experiment involving the uptake and transport of 5,6-CF from source and transition leaves. What was noticed was that prior to any export out of a supply leaf, the fluorophore first entered the opposite leaflet to the fed leaflet, before exiting the petiole and transporting to sink regions of the plant, via petiolar stem phloem. This phenomenon occurred irrespective of photosynthetic state and has

been described as a prerequisite load balancing process, which always preceded export.

4.1.1 Introduction

It has been long established that immature sink leaves import assimilates from source older leaves until they are able to produce sufficient assimilate to sustain growth and in turn, as maturation occurs, they themselves will become a source of assimilates for the next generation of developing leaves. The passage from an entirely heterotrophic, carbohydrate-importing organ to an entirely autotrophic, carbohydrate-exporting organ was termed the sink-to-source transition and occurs in all expanding leaves (Turgeon 1989, Robinson-Beers *et al.* 1990, Gagnon and Beebe 1996a). Therefore, leaves in the transition state are commonly defined as 'maturing' leaves, which import and export assimilates at the same time (Turgeon 1989). Transition leaves thus have a dual function of being both assimilate exporter and importer but these processes are confined to separate source and sink regions within the leaf. These regions have been shown to support bidirectional transport through the petiole and major veins (Larson *et al.* 1972). Bidirectional transport occurs as a result of the simultaneous import into immature basal regions and concomitant export from mature apical regions (Jones and Eagles 1962, Larson *et al.* 1972). Before the leaf is fully expanded, it must undergo a transition from a sink (net carbon importer) to a source (net carbon

exporter) state. As the leaf expands, unloading from the major veins becomes reduced and eventually ceases (Turgeon 1989, Roberts *et al.*1997, Imlau *et al*. 1999, Oparka *et al*. 1999, Wright *et al*. 2003). It has been suggested that the structure and topology of the major veins facilitate export, rather than redistribution of photosynthate produced within the mesophyll of the precociously mature lamina tip (Larson *et al*. 1972). As a direct result of this, those portions of a leaf which do not synthesize sufficient carbohydrate to meet local requirements for growth must import it from other leaves (Penny and Nelson 1970). Once fully matured, a leaf becomes an irreversible exporter and phloem loading and the subsequent export from source leaves is largely irreversible (Turgeon 1989). Matured leaves thus function as the source of minor vein-loaded photoassimilate, which is transported towards the base of the leaf and which enters the long-distance transport compartment of the phloem (Ruiz-Medrano *et al*. 2001).

In dicotyledonous plants, the transition from photoassimilate sink to source status begins shortly after the leaf has begun to unfold (Turgeon 1989). At this stage in development, the major morphogenetic events that determine leaf shape have already been completed. Maturation of the phloem and xylem in the class I and higher-vein orders, which occurs in an acropetal (lamina base to tip) direction, has been reported to be largely complete before the sink-to-source transition begins (Turgeon 1989). In contrast, structural and functional maturation of

the smaller veins proceeds in a basipetal (tip to base) direction as the leaf unfolds. Therefore, a gradient in the degree of leaf maturation exists from the base to the tip of the lamina during the sink-to-source transition (Turgeon 1989). Transport must, therefore, occur simultaneously in opposite directions, at different locations within a transition leaf at any given time (Geiger 1979, Turgeon 1989, Minchin *et al*. 1993).

Given the wealth of information that exists in the literature, the question which has to be faced is simply how to define the state of maturity relative to a quantified age parameter in a developing leaf. The plastochron index has been used to analyse all aspects of plant development including sink-to-source transition. Gagnon and Beebe (1996a) used the plastochron index to study sink-to-source transition in leaves of *Moricandia arvensis* L., though only leaves 6-8 could be used, since the criteria governing PI were only fulfilled within that leaf range (see argument in chapter 3). Gagnon and Beebe's (1996a) findings confirmed other previous studies that transition occurs in a basipetal fashion, and that the transition had an approximate duration of 2.5 plastochrons, or approximately 5.6 days.

In order to be able to assess phloem transport capacity, the phloem mobile fluorophore 5,6-carboxyfluorescein (5,6-CF) was used to investigate the maturation of the first pair of leaflets on the compound

leaves of *P. sativum*, and the transition from sink to source state. Results were analysed in relation to the leaf plastochron index, and the data reported here, is strong evidence that changes in plant structure-function especially of the phloem is directly related to the plastochron index of the leaflets concerned.

4.1.2 Results

The individual leaflets making up the compound leaf of *P. sativum* contain a primary vein and four lateral vein orders (Fig. 4.1). The primary vein runs the length of the leaf, and class II veins develop from it. These class II veins frequently overarch and interconnect at the leaf margins, providing a "loop" between the veins. Figure 4.1 also illustrates that the space between class II veins is often interconnected directly via intersecondary veins, which delimit the broad, large areolar regions within the leaf blade. These intersecondary veins interconnected with the class II veins at frequent intervals in the interstitial lamina zone between successive class II veins. Clearly, the intersecondary vein must have an important load balancing and assimilate distribution function. The class III veins derived from the class II veins subdivide at regular intervals to outline the form of the minor vein network, which comprises the class IV and the class V veins which in turn delimit the areolar regions responsible for unloading and uptake of assimilates, which for many species have been demonstrated to occur predominately from the minor vein network. As

in all other species reported to date, maturation of the phloem and xylem in the primary vein and class II veins occurs in the acropetal (lamina base to tip) direction and is largely complete before the transition from sink to source begins. While the leaf unfolds, structural and functional maturation of the smaller veins proceed in the basipetal (tip to base) direction (Avery 1933, Turgeon 1989).

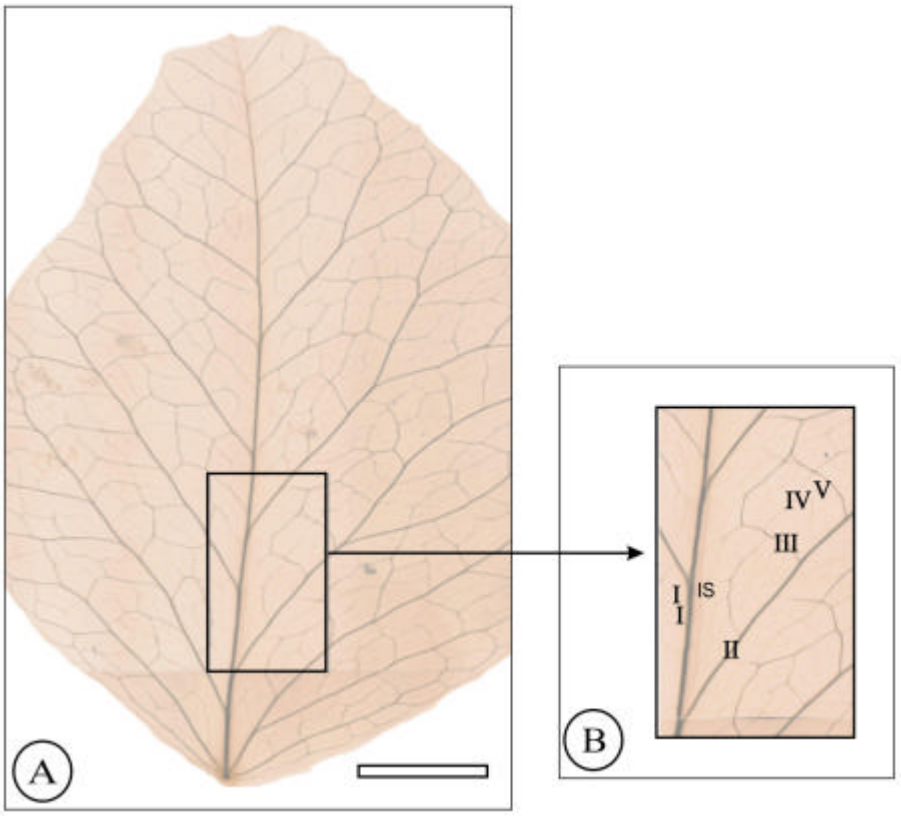

Fig. 4.1: Cleared leaf, stained in safranin to highlight the xylem, which shows the venation pattern in a mature leaflet of Pisum sativum L. (**A**). Detail (B) shows the class I vein (I) and associated connections to a class II, intersecondary (IS), class III, class IV and class V veins. Note that class V veins delimit the leaf areoles within the leaf. Bar in A = 4 mm; bar in B = 1 mm.

Pea leaflets begin to unfold at about LPI -0.5, and are fully unfolded by LPI 0. Full expansion is achieved at LPI 2. At LPI 0, leaflets have attained about 66% of their final size. Figure 4.2A shows the distribution of the fluorescence associated with 5,6-CF in a leaflet at LPI 0. It is apparent that all vein classes visible in this as yet immature and expanding leaflet contain 5,6-CF. Figure 4.2G shows a higher magnification of a portion of the lamina. Some diffusion of 5,6-CF from the class III vein into the mesophyll is evident indicating unloading. It is apparent that the fluorophore is being imported into all regions of the lamina of leaflets at LPI 0. Figure 4.2B shows the reduction of fluorescence in the tip of the leaflets at LPI 0.5. Note that the distribution of fluorescence decreases as the fluorochrome move from the base towards the tip of leaflets. A higher magnification of the tip of the leaflet is shown in Fig. 4.2H. The fluorophore is absent in most veins at the tip and in class III veins where present, they appear threadlike with no unload of the fluorochrome into the mesophyll. A restriction in the flow of the fluorochrome towards the upper region of the leaflet points to the possibility that the region has commenced export as against import at LPI 0.5.

Fig. 4.2C shows the presence of fluorescence only in very few higher vein orders in the upper and most part of the mid regions of leaflets at LPI 1 – 1.5. There is no visible unloading of the fluorochrome into class III veins within these regions. However, fluorescence appear in

the class III veins at the basal region of the leaflet. Note that some diffusion of 5,6-CF into the mesophyll around the class III veins can also be seen. The upper region has ceased to import the fluorochrome completely, while the middle region has commenced gradual transition from import to export. The latter is made evident by the gradient restriction in the flow of the fluorochrome in the upper end of the mid region. However, while the matured upper half of the lamina has ceased to import, the basal half of the lamina is still actively importing 5,6-CF.

Complete absence of fluorescence in the upper, mid and part of the lower regions of the lamina is evident in leaflets at LPI 1.6 – 1.8 as shown in Fig. 4.2D. Reduction in fluorochrome in the class III veins and subsequent diffusion into the mesophyll at the basal region of the leaflets was also apparent. The maturation of the lamina has occurred even further basipetally, capturing the upper end of the lower region of the lamina at LPI 1-6 – 1.8, this signals the commencement of transition of the lower region of the pea leaflets.

Fig. 4.2E shows 5,6-CF in the primary vein as well as in some of the class II veins, up to the mid region of leaflets at LPI 2 while Fig. 4.2F shows that the primary vein in the basal region of the leaflet is fluorescent at LPI 2.3. Fluorescence was not visible within the leaflets by the time they attain LPI 2.5. Unload of the fluorochrome did not

occur in any region of the lamina of leaflets, as the class III veins throughout the lamina, have stopped importing fluorophore completely. This indicates the attainment of maturity by the remaining basal portion of the lamina and thus the maturation of the leaflet.

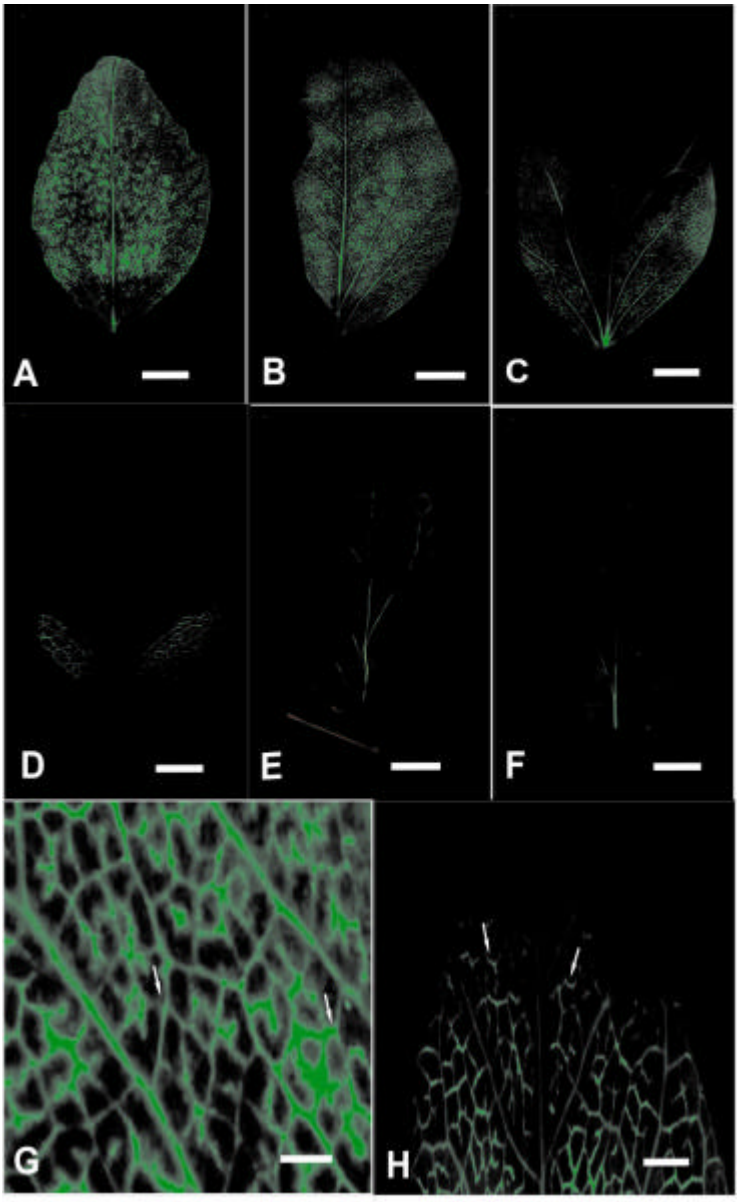

Fig. 4.2: Epifluorescence micrographs which illustrate the distribution of the phloem-mobile probe, 5,6–CF in intact leaflets of P. sativum, 3 h after application of the fluorophore to distant (source) leaflets. Each leaflet is reconstructed from montages of overlapping regions of the leaf surface. Bar in A – F = 4 mm; in G = 40 mm; in H = 16 mm.

Fig. 4.2(A) shows the distribution of 5,6-CF in leaflets at LPI 0. Note that the imported 5,6-CF was transported through all vein groups in all regions of the lamina and once inside the class III vein network, the veins appeared as if "bleeding" (Fig. . 4.2G, arrows) indicating that 5,6-CF was being unloaded into the mesophyll. This pattern of distribution of the fluorophore is typical of a leaflet in the sink state, and was observed in all sink state leaves.

Fig. 4.2(B) shows the distribution of 5,6-CF in leaflets at LPI 0.5. The distribution of the imported fluorochrome in the leaflet is generally similar to that in leaflets in the sink state (Fig. 4.2). Whilst import is evident across the lamina, there was no evidence of the fluorophore towards the tip of the leaflets (Fig. 4.2H, arrows) and where present, it appears threadlike with no evidence of bleeding or thus unloading into the mesophyll; which means the tip is no longer importing. Transition from sink to source has commenced in the tip and is proceeding basipetally in this leaflet.

Fig. 4.2(C) shows the distribution of 5,6-CF in leaflets from LPI 1 – 1.4. The fluorophore was not observed in the tip and mid region of the lamina except in a few higher vein orders, indicating the progressive transition of the upper half of the lamina from sink to source state. Leaflets from LPI 1 – 1.4 are thus considered to be at an advanced sink to source transition state.

Fig. 4.2(D) shows the distribution of 5,6-CF in leaflets from LPI 1.6 - 1.8. Distribution and the unloading of the fluorophore via the class III vein network is restricted and is limited portions of the lower half of the lamina. Leaflets between LPI 1.6 and 1.8 are thus at the late sink - source transition stage.

Fig. 4.2(E) and (F) show the inflow of 5,6-CF into leaflets at LPI 2 and 2.5 respectively. In leaflets at LPI 2, the fluorophore did not move beyond the primary vein and few class II veins (4.2E); movement of the fluorophore into these veins was even more restricted in the leaflet at LPI 2.5 (4.2F). Leaflets at this LPI range, are thus in an early source development state.

4.1.3 Discussion

The results reported in this chapter demonstrate that sink-to-source transition in *P. sativum* can be related to leaf plastochron index (LPI). The gradual cessation of import from the tip to the base of leaflets was studied using the phloem mobile fluorophore, 5,6-CF. In essence, the results confirm those of Gagnon and Beebe (1996a) that sink-to-source transition is directly correlated to leaf plastochron index.

All regions of lamina of leaflets at LPI 0 were shown to import 5,6-CF from a distant source leaflet and to unload it into the mesophyll, via the class III vein network. The class IV and class V veins were clearly not involved in the import process, and as expected, they are only functional during export in this case, as well. Unloading of the fluorochrome by the class III veins was evident through a phenomenon described previously by Roberts *et al*. (1997) as a 'bleeding appearance' of the class III vein network in young *Nicotiana benthamiana* leaves. The import and diffusion of 5,6-CF into the mesophyll in leaflets at LPI 0 are thus a strong indication that the leaflets are still in the sink state.

An acropetal reduction in fluorescence was observed when leaflets reached LPI 0.5, suggesting that a restriction in the flow of the fluorochrome towards the tip was taking place. In all cases, 5,6-CF

was absent at the tip of leaflets at LPI 0.5 but it was still evident in the more distal ends of the class III vein network. It is important to stress that no apparent unloading of the fluorophore into the mesophyll around the veins occurred. Thus, whilst class III veins in the lamina tip region may continue to import 5,6-CF, there is no evidence of unloading associated with these veins. The lamina tips of leaflets at LPI 0.5 have thus transitioned to an export phase. It is therefore reasonable to argue that sink-to-source transition must commence between LPI 0 and 0.5 in the pea variety used in these experiments.

5,6-CF was absent in the apical as well as in parts of the mid region of the lamina by LPI 1 to 1.5. However, very few higher vein orders showed evidence of 5,6-CF fluorescence which suggests that import into the upper half of the lamina was restricted by this stage. In contrast, fluorescence was present in the basal region and unloading of 5,6-CF via the major vein network, is indicative that the basal region of the lamina is still immature and as such is still importing 5,6-CF from the fed distant source leaflets and that the transition from sink to source state had advanced basipetally. Based on the data obtained in these experiments, the leaves which are at LPI 1 – 1.5 are classified as being at an advanced sink-to-source transition stage.

Between LPI 1.6 and 1.8, the transition from sink to source was near-completion in the basal regions. Some evidence of continued

offloading was observed (Fig. 4.2D), but only at the extreme marginal base of the leaflets, signifying the near-completion of the leaflet's transition from sink to source.

However, there was still evidence of the movement of fluorophore into the higher vein orders, up to the mid region of the leaflet in leaflets at LPI 2 and the fluorochrome moved outwards from the primary veins into few class II veins in these leaflets. By LPI 2.3, little evidence of 5,6-CF uptake by the primary vein at the basal region of leaflets could be seen and by LPI 2.5, no fluorescence was evident in the primary vein. The presence of the fluorophore in the primary and class II veins as observed in leaflets which are at LPI 2 – 2.3 is not necessarily an indication of active import as pointed out by Turgeon (1989). According to Turgeon (1989), though there is usually a clear boundary between the importing and non-importing regions, label frequently extends into the non-importing zone in the larger veins. Therefore it is presumed that the larger veins of leaflets at LPI 2 – 2.3 are actually within exporting zones thus, the leaflets at this stage are possibly at an early stage of transition to the source state. This data finds support from the earlier work by Wimmers and Turgeon (1991) who used [14C] sucrose to examine sink-to-source transition in *P. sativum* L. cv. Little Marvel. Wimmers and Turgeon (1991) reported that the transition process occurs between LPI 1.6 and 1.8. The authors however used leaf length in calculating plastochron index as against

average leaflet length used in this study which might account for the discrepancy of approximately 0.4 to 0.5 plastochrons. As such, direct results comparison of Wimmers and Turgeon's (1991) experiments with those reported here cannot be made.

From the literatures it is evident that some variability exists in the number of consecutive leaves which are in sink-to-source transition at any one time. For example, Gagnon and Beebe (1996a) reported that four successive leaves were found to be in transition simultaneously in *Moricandia arvensis* L. Four leaves were also in transition in sugar beet and tobacco (Turgeon and Webb 1973). In contrast, the squash plant has only one leaf at a time which is in sink-to-source transition (Turgeon and Webb 1973). The results of the experiments reported here, reveal that 1-2 leaves are under transition at the same time on the pea plant, at any given time. The duration of sink-to-source transition appears to be species dependent. For example, Gagnon and Beebe (1996a) reported that sink-to-source transition took an approximate duration of 2.5 plastochrons in *M. arvensis* L., while results of the experiments reported in this chapter show that sink-to-source transition in *P. sativum* takes about 2 plastochrons

The data that is presented in this chapter show that sink-to-source transition in pea leaves is directly related to leaf plastochron index. The sink/source state of leaflets at various LPIs, as well as the duration

of the sink-to-source transition process and the number of leaves under transition at a given time may be determined by using the plastochron index.

4.2 A Modular Supply and Load-Balancing Mechanism is a Prerequisite for Export in Compound Leaves of Pea Plants

4.2.1 Introduction

According to Patrick *et al.* (2001), most actively growing regions, as well as storage organs (sinks) of higher plants, import assimilates in solution by bulk flow through the phloem, where movement of assimilate is driven by differences in hydrostatic pressure along the source to sink phloem transport pathway. Furthermore, Patrick *et al.* (2001) stated that osmotic water movement generates the required pressure gradient between the extremities within the phloem transport pathway. There is considerable evidence for leaves becoming exporters once the leaves become fully matured, after which phloem loading and the subsequent export from source leaves is largely irreversible (see Turgeon 1989 and literatures cited). At maturity, leaves function as the source of minor vein-loaded photoassimilate, and assimilate is transported towards the base of the leaf, where it enters the long-distance transport compartment of the phloem. Heterotrophic tissues (that is, sink leaves) receive nutrients either by symplasmic delivery via plasmodesmata, or through the apoplasm via sugar transport systems located on the plasma membranes of the companion-cell–sieve-element (CC–SE) complex and surrounding meristematic or receiver cells (Ruiz-Medrano *et al.* 2001). The relationship between phloem transport and the maturation of the

phloem transport pathway in leaflet pairs in a hypothetical plant is illustrated in Fig. 4.3. Export from pairs of mature source leaves is, I believe, very similar, given that these leaflets are formed at the same time. Phloem translocation and the subsequent delivery of assimilates will be directed acropetally towards the strong sink region which is represented by the sink leaflets. Transition leaflets will progressively lose their sink strength, and assimilate import will decline as the leaflets undergo transition from a sink to a source state and little if any import of assimilate would occur under these conditions. It is logical therefore to expect that in plants with compound leaves, opposite leaflet pairs would have approximately near-identical structural and functional states. It is also expected therefore that leaflets of the same age would transit through sink to source states at approximately the same time as well. The hypothesis is therefore, that a degree of balancing of imports into sink leaflets would be necessary to ensure that leaf expansion and maturation proceeded in synchrony. In order to be able to test the hypothesis experimentally, transport of the phloem-mobile fluorophore 5,6-carboxyfluorescein (5,6-CF) was studied by loading one of the pair of leaflets and examining the subsequent transport state of the leaflets.

The aim of the experiments reported in this second part of chapter 4 was thus to determine if assimilates are transported between paired leaflets of the compound leaves of pea plants.

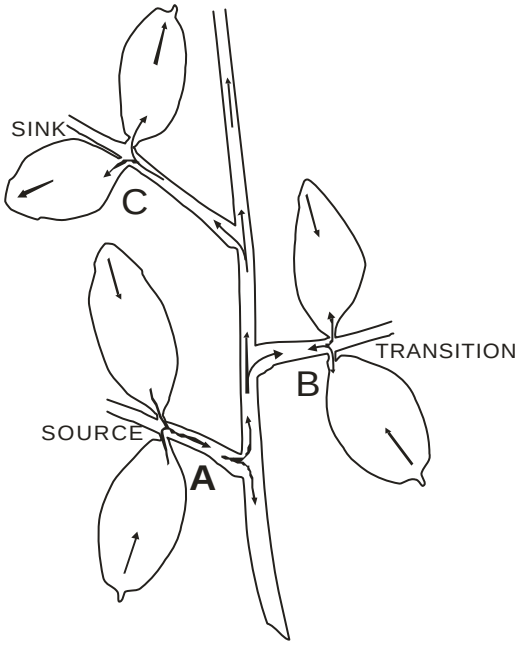

Fig. 4.3: Shows the hypothetical phloem stream functioning within a plant with compound leaves. The paired leaflets are assumed to be of same age and therefore at the same sink/source state, with similar assimilate flow pattern. Assimilates move from source leaflets (A), into the main assimilation stream within the stem and thereafter are taken up by importing regions and out of exporting regions of transition leaflets (B); movement is inwards in sink leaflets (C).

4.2.2 Results

Having established the source to sink transition characteristic of the Greenfeast variety in the first part of this chapter, a series of experiments were set up in order to investigate transport of the tracer in the phloem from a leaflet within the first pair of leaflets of sink, transition and source leaves to the opposite leaflet. The transport characteristics of the fluorophore were examined in the leaflet opposite the fed leaflet, 30 min to 3 h after application, by examining the whole leaflets from their abaxial surface using the Olympus BX-61 epifluorescence microscope. Images of leaflets from the earlier study in which 5,6-CF was loaded from a distant source leaflet (Fig. 4.4A-C), were compared to those at similar LPI used in the present study (Fig. 4.4D-F).

In sink leaflets, 5,6-CF was transported across the node and unloaded via the primary vein to the class II and class III veins in the leaflet. Unloading from the class III veins to the mesophyll was routinely observed. No export out of the leaf axis was observed (Fig. 4.4D). In transition leaflets, 5,6-CF first transported across the node into the primary vein and into lower (i.e. first and some (more basal) class III veins (Fig. 4.4E). Unloading was observed to be dependent on the state of transition of the leaflet pair, in other words, the plastochron age of these leaflets. The fluorophore was thereafter exported out of the transition leaflet via the petiole. Where a source leaflet was offered 5,6-CFDA, the 5,6-CF first transported across the node interlinking the

leaflet pair, to the opposite leaflet. Distribution of the fluorophore always occurred via the primary vein, to the class II vein up to or near the tip of the leaflets and the leaflet margins, via the class II veins (Fig. 4.4F). No unloading was observed in the leaflet mesophyll. The fluorophore exited the leaflets afterwards.

When distant source leaves were offered 5,6-CFDA as discussed in the first part of this chapter, accumulation of 5,6-CF in paired sink or transition leaflets were typified by results shown in Fig. 4.4A and 4.4B for sink and transition leaflets i.e. 5,6-CF entered both leaflets.

Experiments carried out by applying 5,6-CFDA to predarkened leaflets gave the same results as those undertaken with leaflets that had been exposed to continuous light. In these experiments, 5,6-CF first moved from the predarkened fed leaflet into the opposite leaflet (which was maintained in continuous light) and thereafter exited the leaflets via the petiole in source and transition leaflets.

Fig. 4.5A shows a nodal complex between mature source leaflets. The cross-connection of the axial phloem strands via a well-developed vascular bridge is evident in this micrograph. Figure 4.5B is a longitudinal section of a nodal complex which shows strong 5,6-CF-associated fluorescence within the phloem strands which interconnect a source leaflet pair. The leaflet to which 5,6-CFDA was applied (the

fed leaflet) was attached to the right. In this example as in all other experiments, 5,6-CF first moved via the interconnecting phloem bridge to the opposite leaflet, before exiting the leaf via the axial phloem strands.

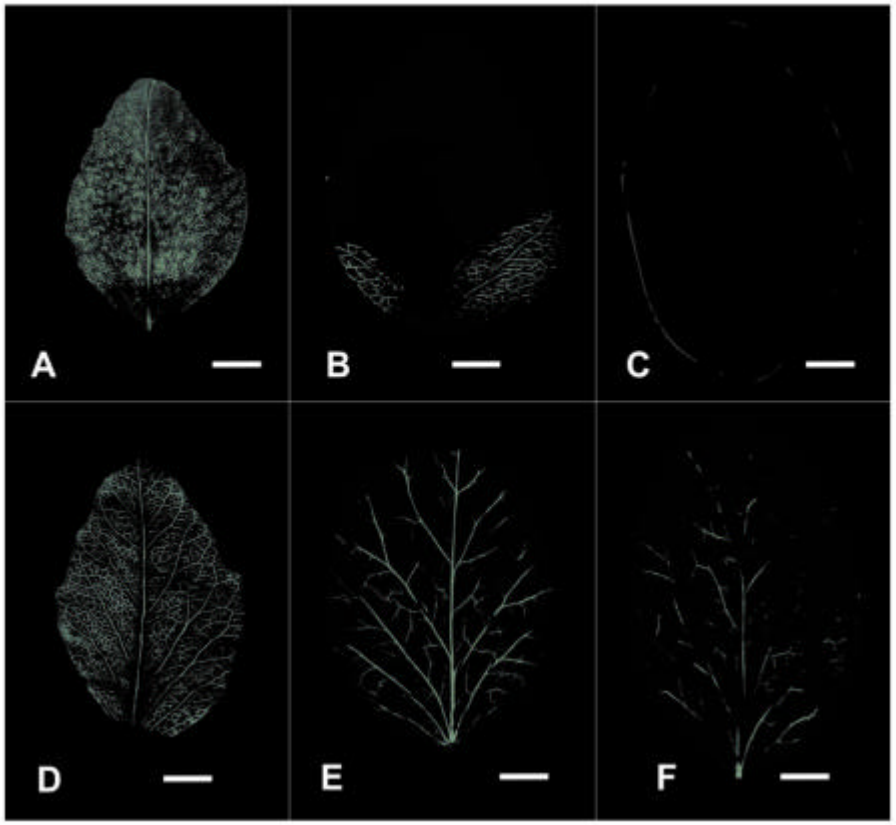

Fig. 4.4: Epifluorescence images showing the transport and distribution of 5,6-CF via the phloem in intact leaflets of P. sativum, 3 h after application of the fluorophore from distant (source) and local (opposite) leaflets. Each leaflet was reconstructed from montages of overlapping regions of the leaf surface. Bars in A-F = 4 mm.

Fig. 4.4(A) shows a typical sink leaflet in which the 5,6-CF imported from the fed distant source leaflets was distributed throughout the lamina with high labelling of the class III veins.

Fig. 4.4(B) shows a typical transition leaflet in which the class III veins in the immature basal portion of the lamina are still functional as an import pathway for the 5,6-CF from the fed (distant source) leaflets while the apical region has ceased import, thus no 5,6-CF is evident in this region.

Fig. 4.4(C) shows a typical mature source leaflet in which no 5,6-CF is evident. No import of 5,6– CF from the fed distant source leaflet occurred.

Fig. 4.4(D) shows the distribution of the 5,6-CF within an attached sink leaflet paired to the opposite sink leaflet to which 5,6-CFDA was applied. The fluorophore is evident throughout the lamina with high labelling of the class III veins, in a pattern similar to that in Fig. 4.4A.

Fig. 4.4(E) shows the distribution of the 5,6-CF within an attached transition leaflet paired to the opposite transition leaflet to which 5,6-CFDA was applied. In contrast to the pattern in Fig. 4.4B, the fluorophore was transported up to the extremities of the major veins and into the class III veins in the basal, less mature regions of the leaflet. Thereafter, the fluorophore was exported via the petiole.

Fig. 4.4(F) shows the distribution of the 5,6-CF within an attached source leaflet paired to (opposite) a fed source leaflet. The fluorophore was transported by and restricted to, the major veins of the leaflet before exiting via the petiole; in contrast to Fig. 4.4C where no evidence of the fluorophore was observed.

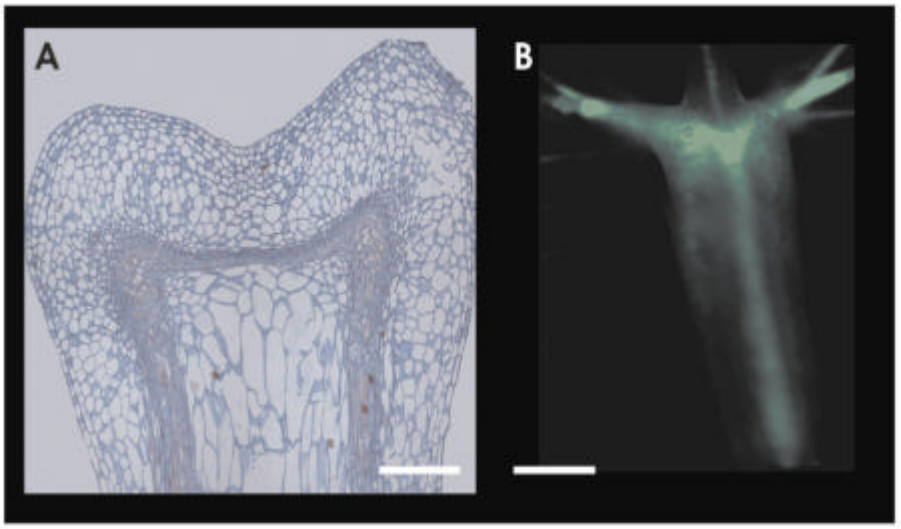

Fig. 4.5: Shows details of the anatomy (A) and transport of 5,6-CF (B) across the petiole between paired leaflets in longitudinal section.

Fig. 4.5(A) shows the petiole traces to a leaflet pair in longitudinal section. Note that the axial traces are cross-connected via a strongly developed lateral vascular trace. Bar = 1.0 mm.

Fig. 4.5(B) 5,6-CF moved from the site of application (to the right) and crossed to the opposite leaf trace via the cross-connection. Some evidence for axial transport is evident. Bar = 0.5 mm.

4.2.3 Discussion

The experiments using connected, attached leaflets of the pea plant convincingly demonstrate that the fluorophore 5,6-CF, was first transported to the opposite leaflet before any export occurred. Results reported in this chapter have demonstrated that 5,6-CFDA is taken up by the leaf and that the cleavage product 5,6-CF is transported to, and is subsequently taken up by the phloem tissue in pea leaves and thereafter, that it is transported via the phloem, following classical source to sink transport rules. The phloem-mobile fluorophore was transported along a source to sink pathway, common with any substance which can (a) enter the phloem and (b) which can be transported passively within the symplasmic translocation pathway. Of greater significance is that the data offer convincing evidence for the existence of a load-balancing mechanism between leaflet pairs in compound leaves such as in *P. sativum*.

Functional cross-connections exist within the nodal complex at the base of leaflet pairs in compound leaf of peas, and that this nodal complex is in turn, directly connected to the axial phloem system within the petiole itself. These cross-connections appear therefore to provide the phloem infrastructure necessary for an operative load balancing mechanism. Connectivity across the petiole via subsidiary traces may thus provide a functional channel through which assimilate

(or phloem sap) concentrations in oppositely-positioned leaflets are maintained at equal, or near equal sap concentration, during import to, or export from leaflets via the axial traces.

The data demonstrate unequivocally that local source 5,6-CF uptake and its subsequent translocation into sink leaflets, as well as its uptake into transition and source leaflets, is consistently preceded by movement first into the opposite leaflet before any axial transport takes place. Of interest is the appearance of two distinct phloem transport pathways into transition and source leaflets. If the source of assimilates in transition leaflets is local (i.e. the opposite leaflet of the pair), then import (as evidenced by the passively-transported 5,6-CF) is via the major veins (primary and class II) veins to the extremities of the veins, as well as in class III veins in the basal, less mature regions of the transition leaflets, which is quite evident in Fig. 4.4E. In contrast, Fig. 4.4B shows the phloem transport pathway of 5,6-CF, where the source of 5,6-CF is distant. Here the 5,6-CF enters the opposite leaflet and is confined to the lateral veins (class II and class III vein network) in the basal less mature region of this transition leaflet. When one of a pair of source leaflets was offered 5,6-CFDA, the 5,6-CF was transported to the opposite leaflet via the major veins. Note however, that no evidence of 5,6-CF appeared in same leaflets where 5,6-CFDA was applied at a more distant source leaf. Clearly, local application of 5,6-CFDA to sink, transition and source leaflets

gave consistently different results which I interpret as a local balancing, local transport system which ensures near equal concentration of assimilate in leaflet pair.

The results find support in the earlier results obtained by Isebrands and Larson (1977) who conclusively demonstrated that subsidiary vascular bundles provided vascular continuity between the stem and specific portions of the leaf lamina in Eastern cottonwood. However, as tempting as it may be, to speculate that a bi-directional modality exists within the vein in leaflets, it is important to stress that this has, as yet, not been demonstrated to be the case for a single file of sieve tubes (Turgeon 1989, Henton *et al*. 2002). The differences in the pattern of distribution of 5,6–CF transported from distant sources into sink, transition and source leaflets (Fig. 4.4A-C) compared to that seen in local sources in leaflets of same physiological state (Fig. 4.4D-F), show that the vascular link between the stem and the lamina is different from the vascular crosslink between the paired leaflets. The strong and consistent lateral movement of the fluorochrome out of fed leaflets, and its subsequent appearance in the opposite leaflets, irrespective of the sink/source state of the cross-connected leaflets, is strong evidence that transport across vascular connection between leaflets is autonomous, and independent of the relationship between the leaflet pair in question and other compound leaves along the phyllotaxy or also independent of the source to sink gradient.

Plants have previously been described as modular organisms, consisting not only of morphological and of physiological subunits, which act autonomously in part and are thus termed “functional modules” or “integrated physiological units” (IPUs) that have their own carbon source or supply (see Watson and Casper 1984, Watson 1986). A modular structure has important consequences for the control of internal resource translocation and distribution in plants, and therefore in the regulation, by partitioning, of differential growth of plant parts. Indeed, some plant parts may act as partially autonomous functional modules with their own resource supply and localized control of growth (Kaitaniemi and Honkanen 1996). Yang and Midmore (2004) have proposed a model in which the transport process is included as a significant component of the modular nature of growth. Yang and Midmore (2004) argue that because of the distance and complex structure and function of the transport pathway, each foliage branch takes (first) priority for the allocation of the carbon that it fixes, thereby satisfying its own requirement for assimilate despite fluctuation in supply, before any excess assimilate is transported to other parts of the plant.

In conclusion, the data offer strong support to the concept of modular transport and the evidence which is presented here for reallocation of and balancing of assimilate across leaflet pairs takes place to ensure

that leaflets of the same physiological age, maintain similar or near-identical transport status, thereby utilizing assimilates first, before any export can or will take place. I argue that load balancing of the local transport system before exporting is thus a prerequisite before export to other younger (sink) regions can be take place. I argue further that the model for describing phloem transport into and out of plants bearing simple leaves is inappropriate to the situation in compound leaves such as *P. sativum* (Fig. 4.3). The interpretation of phloem transport across paired leaflets (Fig. 4.6) suggests that phloem transport between leaflet pairs is independent of the sink/source state of the leaflets, or of movement along the source to sink gradient.

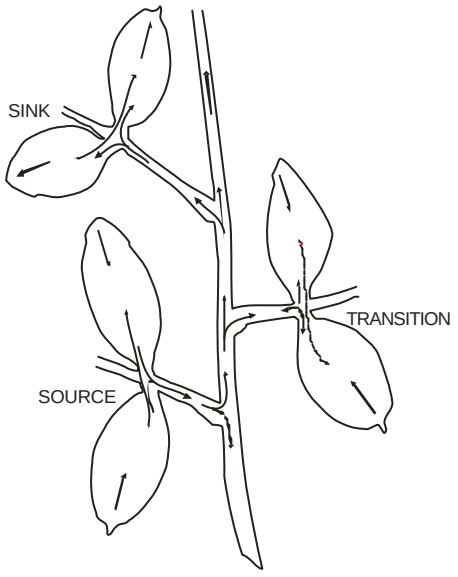

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Fig. 4.6: Illustration of the assimilate pathway through the phloem of a plant with compound leaves, as interpreted from the results. Accumulation patterns of 5,6-CF suggests that phloem transport takes place between paired leaflets (dotted lines), irrespective of the sink/source state of the leaflets; and it is independent of the movement along the source to sink gradient (bold lines).

4.3 Is the movement of 5,6-CFDA through the xylem or the phloem?

It is possible that some damage could have occurred to the xylem during the abrasion process, to expose underlying xylem to 5,6-CFDA. If so, it is likely that some 5,6-CFDA could have been taken up in the transpiration stream directly. If 5,6-CFDA would be transported passively in the apoplast along the transpiration stream, it would arrive rapidly at the sink. The xylem in vascular bundles of the leaf is surrounded by living parenchyma cells, which are connected to the xylem through pits which are lined by the plasmamembrane on the pore side of the pit. The pit membrane is fibrous and permeable on the xylem side and plasmamembrane is selectively permeable on the parenchyma side. It has been shown that 5,6-CFDA can offload through the pore from the xylem into the parenchyma cells. Once the 5,6-CFDA has been transported across the pit-plasmamembrane interface, the acetate moieties are cleaved by acetyl CoA and 5,6-CF is released. 5,6-CF is not capable of crossing membranes and cannot move out from the parenchyma cells back into the transpiration stream. 5,6-CF can only cross to adjacent living cells if plasmodesmata are present, and could conceivably be taken up by the phloem tissue along a symplasmic route (Personal communication, CEJ Botha)

Offloading of 5,6-CFDA from the xylem into the parenchyma cells could occur anywhere along the translocation stream and is therefore

not necessarily confined to distant sink tissues, the transpiration stream is dependent on transpiration rate and is not source-sink dependent. If 5,6-CFDA is cleaved in the xylem, in other words, 5,6-CF not 5,6-CFDA is transported in the transpiration stream, logically one will expect to find 5,6-CF in all terminal veinlets within the leaflet, irrespective of them being source or sink. Botha *et al*. (2005) have shown that where 5,6-CFDA is allowed to move into cut leaves, it is usually transported along the transpiration stream (xylem vessels) and offloaded via parenchyma cells in all regions of the lamina. Clearly, this is not what was observed in my experiments, as only some class III veins dependent on the sink/source status contain evidence of 5,6-CF (Fig. 4.2 and 4.4). Results of the experiments showed that 5,6-CF is restricted to (if allowed into) certain regions of the lamina when coming from a distant source (Fig. 4.2B-D) and even more restricted to the higher order veins in transition and source leaflets, when coming from a local (opposite) source (Fig. 4.4E and F). This would seem to rule out the idea that significant quantities of 5,6-CFDA were transported through the xylem (which is present in all sink and source leaflets) at least in the leaflets presented in these reports. It is apparent that 5,6-CF is within vascular parenchyma cells and possibly sieve elements.

As regards movement of 5,6-CF between paired leaflets, in order for 5,6-CFDA to be transported via the xylem across to the opposite

leaflets, it would have to move against the transpiration stream in transpiring attached leaflets. It is unlikely that this could occur in such a short period of time (10 min) as reported in this study. Furthermore, it should be noted that 5,6-CF first appears in the leaflet opposite the fed leaflet, before any basipetal movement occurs.

4.4 Evidence for the existence of a symplasmic pathway in *P. sativum* L.

Like other members of the Fabaceae, the minor veins of *Pisum* are categorized as a closed system termed 'type 2 minor vein' configuration due to the presence of few or no plasmodesmal connections between the sieve element-transfer cell complex (SE-TCC) and the adjacent cells (Gamalei 1989, van Bel and Gamalei 1991). *Pisum* is classified further into the category of type 2'b' minor vein configuration due to the presence of transfer cells with the characteristic wall ingrowths in the minor vein phloem (Gamalei 1989). According to van Bel *et al*. (1992), there is a correlation between minor vein configuration and phloem loading. By reason of low plasmodesmatal frequency, the pathway of the flow of assimilates in plants with type 2 minor vein configuration is considered to be apoplasmic (Gamalei 1989, van Bel and Gamalei 1991). Wimmers and Turgeon (1991) have shown that wall ingrowths in minor vein transfer cells of *P. sativum* L. facilitate uptake of photoassimilate by increasing plasmalemma surface area as proposed by Gunning *et al*.

(1968) in their study of transfer cells of *P. arvense*. According to Gunning *et al*. (1968) two contrasting specializations that are likely to favour efficient absorption of materials into a cell are: (1) enhancement of symplasmic transfer through the development of abundant plasmodesmata and (2) the promotion of capacity uptake from the extracellular environment though increase in the cell's surface:volume ratio. The transfer cells of *P. sativum* are supposedly specialized in the latter direction like those of their counterpart in *P. arvense* (Gunning *et al*. 1968). However, Gunning *et al*. (1968) did not rule out the potential for symplasmic transfer in *P. arvense* via the branched plasmodesmata traversing the wall between transfer cells and their associated sieve elements and also those interconnecting the transfer cells; and since phloem loading is a family-specific multiprogrammed mechanism (van Bel and Gamalei 1991), transport via symplasmic pathway cannot also be ruled out in *P. sativum*. This is justified even the more, with the results of the experiments reported in this chapter.

It has been pointed out that plasmodesmatal frequency alone does not make an absolute case for either symplasmic or apoplasmic transport; more importantly because the minimum frequency required for symplasmic movement is yet unknown (Fisher 1986, Warmbrodt and van der Woude (1990), Botha and van Bel 1992). Botha and van Bel (1992) have argued that, plasmodesmata, whether presented as tables or plasmodesmograms, give an indication of the maximum potential

pathway of symplasmic transport. They argued further that, frequencies merely represent the number of plasmodesmatal connections determined at a particular time, for a particular interface and at a particular stage of development of the cells making up the interface. According to the authors, plasmodesmatal frequency per se does not take the transport capacity of the plasmodesmata into account.

The hypothesis has been that plants with the type 2b minor vein configuration translocate assimilates mainly through the apoplasmic pathway have been demonstrated in *P. sativum* L. (Turgeon and Wimmers 1988, van Bel *et al*. 1992) and other apoplasmic species (van Bel *et al*. 1992), through the use of the apoplasmic blocker p-chloromercuribenzenesulfonic acid (PCMBS) to block the loading of $^{14}CO_2$. The experiments gave credence to a correlation between minor vein configuration in terms of plasmodesmata frequency and phloem loading pathway. Wimmers and Turgeon (1991) showed that plasmodesmata frequency between the SE-TCC and surrounding cells is low in *P. sativum* and that this cast doubts on the efficacy of a symplasmic pathway in the plant. However, experiments have shown that in any species, the frequency of plasmodesmata is expected to decline from the mesophyll toward the minor-vein phloem to compensate for changes in the surface-area to volume ratio of various tissue types even if the pathway of solute diffusion is entirely symplasmic (Ding *et al*. 1988, Turgeon and Beebe 1991), therefore

Wimmers and Turgeon (1991) agreed that plasmodesmata frequency in the SE-TCC boundary is, in itself, not convincing evidence for an apoplasmic pathway.

Carboxyfluorescein was developed as an advanced form of fluorescein; because though the latter enters the symplast and is transported in the phloem (Schumacher 1933 in Wright and Oparka 1996), it leaks through the plasma membrane at an appreciable rate due to its partial non-dissociation at physiological pHs (Socolar and Loewenstein 1979 in Wright and Oparka 1996). With the presence of an additional carboxyl group on either the 5 or 6 positions on the carboxyfluorescein (CF) molecule, the entry and restriction of 5,6-CF to the symplast and transportation within the phloem is ensured as it becomes less permeant to biological membranes than fluorescein (Wright and Oparka 1996). 5,6-CF has been shown to be transported in this same pattern as 14C assimilates, thus making it an effective phloem-mobile tracer (Grignon *et al*. 1989). When a lamina is loaded with the non-fluorescent ester, 5.6-CFDA, the compound will passively cross the cell membranes into the electrically neutral or near-neutral form. Once inside the cells they are subject to cleavage by esterases to form the polar fluorescent compound, 5,6-CF. 5,6-CF is then transported within the symplasmic pathway of the phloem (Wright and Oparka 1996).

According to Wimmers and Turgeon (1991), the relative paucity of plasmodesmata connecting the sieve element-transfer cell (SE-TC) complex to surrounding cells in a plant like *P. sativum* L., in comparison to species in which the companion cells are not specialized as transfer cells, indicates that the uptake of sucrose by the SE-TC complex is primarily, if not entirely, apoplasmic. However, the results of the experiments reported in here showed the transport of 5,6-CF only within the major and class III veins of importing leaflets. Figure 4.2H clearly shows that the unloading of the fluorochrome into the mesophyll was through the class III vein network. The transport and unloading of carboxyfluorescein by the class III vein network has also been demonstrated in *Nicotiana benthamiana* by Roberts *et al*. (1997). However, no surprise at such movement is expressed, since the plant has a symplasmic minor vein configuration. However, according to Oparka and Santa Cruz (2000), the postphloem symplast does not place major constraints on the exit of solutes or small proteins from the SE-CCC. Oparka and Santa Cruz (2000) pointed out that various experiments (see literature therein) have shown that macromolecules like fluorescent solutes, radioactive solutes, GFP and systemic RNA signals all exit the phloem in the same pattern as plant synthesized proteins through the major veins, with the class III vein being the most implicated. Unloading from the major veins has been shown to become reduced and eventually ceases as leaves transit from sink to source (Turgeon 1989, Roberts *et al*. 1997, Imlau *et al*. 1999, Oparka

et al. 1999, Wright *et al*. 2003). Oparka *et al*. (1999) have demonstrated that the decrease in the transport and unloading of macromolecules via the symplast during sink-to-source transition is as a result of the decrease in plasmodesmal permeability due to the development of single form plasmodesmata into branched forms. Most of the photoassimilates imported by sink leaves is unloaded by moderately large (major) veins and the smallest (minor) veins are relatively or completely unimportant in this regard (Turgeon and Webb 1976), however, the structure and topology of the major veins have been found to actually facilitate export, rather than redistribution of photosynthate produced by the precociously mature lamina tip during the sink-to-source transition (Larson *et al*. 1972).

The experiments reported here demonstrate conclusively that the loading and resultant export of the fluorescent probe through the phloem of major veins in source pea leaflets and the consequent import and unloading through similar major veins of sink and transition leaflets, through the symplasmic pathway despite the so called low frequency of the plasmodesmata within the phloem of *P. sativum* plants. Interestingly, Grignon *et al*. (1992) have demonstrated the movement of 6(5)carboxyfluorescein (6CF) from source to sink regions (leaves and roots) of *Glycine max*, by showing the transport of the fluorochrome through the phloem of the pulvinus at the base of the petiole. Although Grignon *et al*. (1992) did not show the distribution

of the fluorescent probe in the leaflets of *G. max*, the results reported in this Chapter still find support in their work; since *Pisum* is in the same family as *Glycine* and as stated earlier phloem loading is a family-specific multiprogrammed mechanism (van Bel and Gamalei 1991) and more so, the fluorescent image of the petiole of leaves used in this experiment showed similar distribution of 5,6-CF in the phloem.

It is imperative to conclude that though export through the minor veins of *Pisum* may be via the apoplasmic pathway, the evidence presented in this reports point to the existence of a symplasmic pathway in *P. sativum* L, which invariably the functioning of the plasmodesmata that were observed in all cell-cell interface from the mesophyll cells to the SE-TC complex. (Chapter 5, Fig. 5.4).

CHAPTER 5: Anatomical Investigation of Leaflet Development based on Leaf Plastochron Index

5.1 Introduction

5.1.1 Leaflet ontogeny

The development and maturation of foliage leaves is a complex process, involving many interconnected and interdependent stages. Leaf development commences with a rapid increase in length to project a prominent buttress, which contains a primary vein (midrib) with associated lateral bulges on opposite sides, as the leaf begins to define its characteristic shape. Expansion of the lamina is closely followed by the development of the lateral vein system. These become interconnected first at the tip and then are formed successively toward the less mature base of the leaf (Avery 1933). Cell division ceases first in the epidermis, followed by the middle and lower mesophyll, and then the palisade (Avery 1933). The middle mesophyll layers remain potentially provascular, and all lateral veins, large or small, differentiate from this middle mesophyll. Cell division ceases in the lateral veins and then in the lower mesophyll regions after differentiation is completed in the epidermal layers. The tip thus matures early, but growth continues basipetally as long as the leaf continues to develop. The result of the programmed cell division and differentiation steps is that there is a distinct age-gradient from the

mature distal end of the leaf to its actively growing base, where it is attached to the axis (Avery 1933).

Maturation of the major veins occurs in the opposite direction to that of the minor veins, that is, they differentiate and mature acropetally, in contrast to the minor veins, which mature basipetally (Turgeon 1989). From the literature it is clear that most of the photoassimilates imported by sink leaves must be unloaded by moderately large (major) veins and the smallest (minor) veins are relatively or completely unimportant in this regard. As a result, maturation of the phloem and xylem in the class I and higher-order (smaller) veins is largely complete before the sink-to-source transition begins (Larson *et al.* 1972). However, structural maturity and maturation of the minor veins is thought to coincide with the termination of import (Ding *et al.* 1988). Perhaps, this is due to the fact that the minor veins are involved in the uptake from the mesophyll, and thus form the distribution network to the transport (major) veins within the leaf, when export commences (Turgeon 1989). Differences have been shown to exist between the phloem ultrastructure of the source, sink and transition regions. These differences are, according to Fellows and Geiger (1974), indications that the maturity of the vascular system is in some way related to the import or export status. In support of this argument, the maturation of the phloem in the minor vein network has been

shown to involve further development and maturation of the companion cells and phloem sieve tubes (Turgeon 1989).

5.1.2 The development and maturation of the minor vein phloem

The maturation of the phloem in the minor vein network has been shown to involve the further development and maturation of the companion cells and the phloem sieve tubes (Turgeon 1989), to the point that the phloem becomes fully functional.

5.1.2.1 Companion / transfer cells

The companion cell is regarded by many workers to be a highly specialized parenchymatous cell, which is derived from the same mother cell as its associated phloem sieve tubes and the companion cells have numerous cytoplasmic connections with the sieve tube. The companion cells are distinguished structurally from other parenchymatous cells by their dense, organelle-rich protoplasts and their numerous branched plasmodesmata connecting them to the phloem sieve tubes which are called pore-plasmodesmata. The companion cell remains functionally alive only as long as its phloem sieve tube does (Evert 1990, Gagnon and Beebe 1996b). The companion cell and sieve element thus form a unit - the sieve element – companion cell complex (SE-CC complex, Oparka and Turgeon 1999), which is recognised as the major site of phloem loading in minor veins (Stadler *et al*. 1995, Truernit and Sauer 1995, Gagnon and Beebe 1996b). Some companion cells become highly modified, and

included in these are cells with noticeable wall ingrowths. Companion cells with wall ingrowths are called 'transfer cells' (Pate and Gunning 1969), which are associated with areas where active short-distance transport of solutes takes place (Gunning and Pate 1969, Pate and Gunning 1972, Esau 1977). The development of wall ingrowths in transfer cells begins with the onset of solute flux (Gunning and Pate 1969, Folsom and Cass 1986). Leopold and Kriedemann (1975) stated that wall ingrowths increase the surface-to-volume ratio by an order of magnitude compared with smooth-walled cells, suggesting an enhanced capacity for solute exchange. Wall ingrowths help to increase the capacity of solute flux between the apoplast and the symplast (Gunning and Pate 1969). The transfer cells in *P. sativum* have been classified by Gunning and Pate (1969) as "A" type transfer cells. These are transfer cells that are primarily associated with the phloem, and have wall ingrowths along the entire perimeter, except in the portion of the wall abutting the phloem sieve tube, where the ingrowths are relatively few. In most cases, wall ingrowths develop as finger-like projections, averaging 0.2 µm in diameter and up to 2 µm in length (Leopold and Kriedemann 1975).

Transfer cells of minor-vein phloem have been shown to develop only after export of photoassimilate begins in *P. arvense* (Gunning *et al.* 1968) and *P. sativum* (Peterson and Yeung 1975). Wimmers and Turgeon (1991) have demonstrated that wall ingrowths in minor vein

transfer cells of *P. sativum* facilitate uptake of photoassimilate by increasing plasmalemma surface area as originally proposed by Gunning *et al*. (1968) in their study of transfer cells of *P. arvense*.

5.1.2.2 Phloem sieve tubes

Phloem sieve tubes are considered to be mature, when characteristics representative of complete differentiation can be distinguished. These are an open lumen, the absence of a nucleus and ribosomes, and the presence of specialized plastids (Gagnon and Beebe 1996b). The lumen of phloem sieve tubes becomes clears and open to accommodate the translocation stream (Oparka and Turgeon (1999).

Fellows and Geiger's (1974) work on sugar beet demonstrated that the development of mature, relatively open sieve tubes, like those involved in phloem loading, is a preparatory step and does not itself trigger the onset of export. Gagnon and Beebe's (1996b) study of importing *Moricandia arvensis* leaf tissues showed the presence of few mature phloem sieve tubes in the relatively large minor veins (class IV and V) while none were observed in the smallest (class VI) veins. Gagnon and Beebe (1996b) acknowledged that phloem maturation is not closely correlated with import termination. However, the authors observed that the number of mature phloem sieve tubes increased in non-importing tissue just distal to the import termination boundary and especially in apical regions and therefore concluded that there must be

an association in some way between phloem maturation and import termination.

5.1.2.3 Plasmodesmata

Gagnon and Beebe (1996b) suggested that there was no apparent correlation between the development of the plasmodesmata in minor veins and sink-to-source transition in plants with an apoplasmic minor vein configuration. Earlier, Beebe and Evert (1992) and Evert *et al*. (1996) have argued that the lack of a clear correlation between plasmodesmal development and sink-to-source transition could be a reflection of the secondary role of plasmodesmata in apoplasmic phloem loading. According to the authors, if plasmodesmata at the companion cell-contiguous cell interfaces serve as a barrier to symplasmic leakage, their differentiation would most likely serve as a preparatory step for the initiation of the phloem-loading capacity, rather than as a triggering step. The authors suggested that the obstruction of the plasmodesmal branch on the companion cell side may also be involved in cessation of import by disrupting the symplasmic unloading pathway in sink tissue. However, Gagnon and Beebe (1996b) reiterated that the fact that maturation of the specialized plasmodesmata is not directly correlated with the arrest of assimilate import seemingly precludes this possibility.

From the above discussion, it should be clear that whilst a great deal is known about the development and differentiation of lamina cross

section and its minor vein phloem. However, few, if any, of these studies have been carried out where development was examined based on a time sequence. The experiments which are reported in this chapter, deal with the investigations of the development of the internal structure of the lamina and the maturation of the minor vein phloem, correlated to leaf plastochron index (LPI).

5.2 Results

Figs 5.1 A – C show the changes that occurred within mesophyll cells in leaves between LPI 0 and 1. What is apparent is that the palisade mesophyll cells elongate between LPI 0 (Fig. 5.1A) and 0.5 (Fig. 5.1B). The spongy mesophyll intercellular spaces become apparent progressively between LPI 0 (Fig. 5.1B) and 1 (Fig. 5.1C). Few intercellular spaces are also evident in the palisade layer at LPI 1 (Fig. 5.1C). More chloroplasts were evident with increase in LPI. The leaf blades appear thicker with increase in LPI. Further changes in the different layers that make up the lamina were not observed after LPI 1.

Fig. 5.2A shows transverse sections of mature class V vein containing xylem and phloem cells surrounded by a bundle sheath in tissues at LPI 2.0. The phloem sieve tubes are mature and transfer cells contain wall ingrowths. Figure 5.2B shows several minor veins at different stages of development in tissues at LPI 0. The class IV veins contain both mature (open arrow heads, Figs. 5.2 A,B) and immature (solid

arrow head Fig. 5.2A) phloem sieve tubes while the class V vein (circled) is still undergoing division and invariably contains immature phloem sieve tubes. Figure 5.2C shows minor veins at different stages of development in tissues at LPI 1. The phloem sieve tubes are mature.

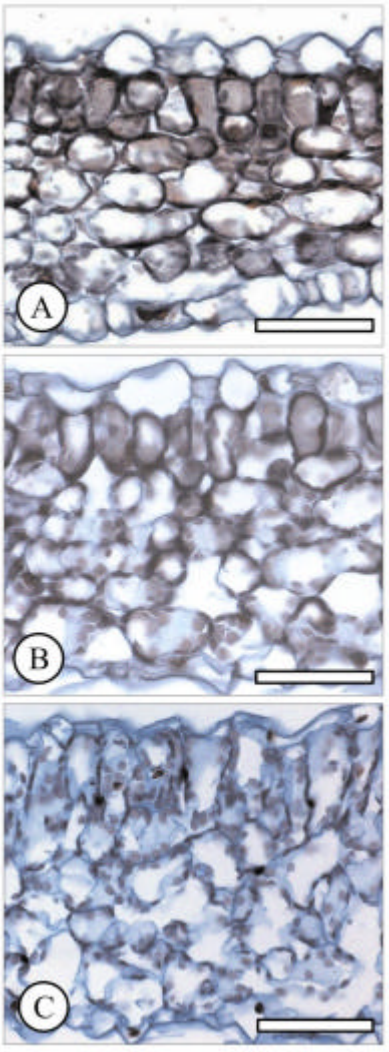

Fig. 5.1: Transverse section of the lamina of leaflets at LPI 0 (**A**); LPI 0.5 (**B**); LPI 1 (**C**). Note the enlargement of the intercellular spaces and increase in chloroplasts number as leaflets age from LPI 0 to LPI 1.0.

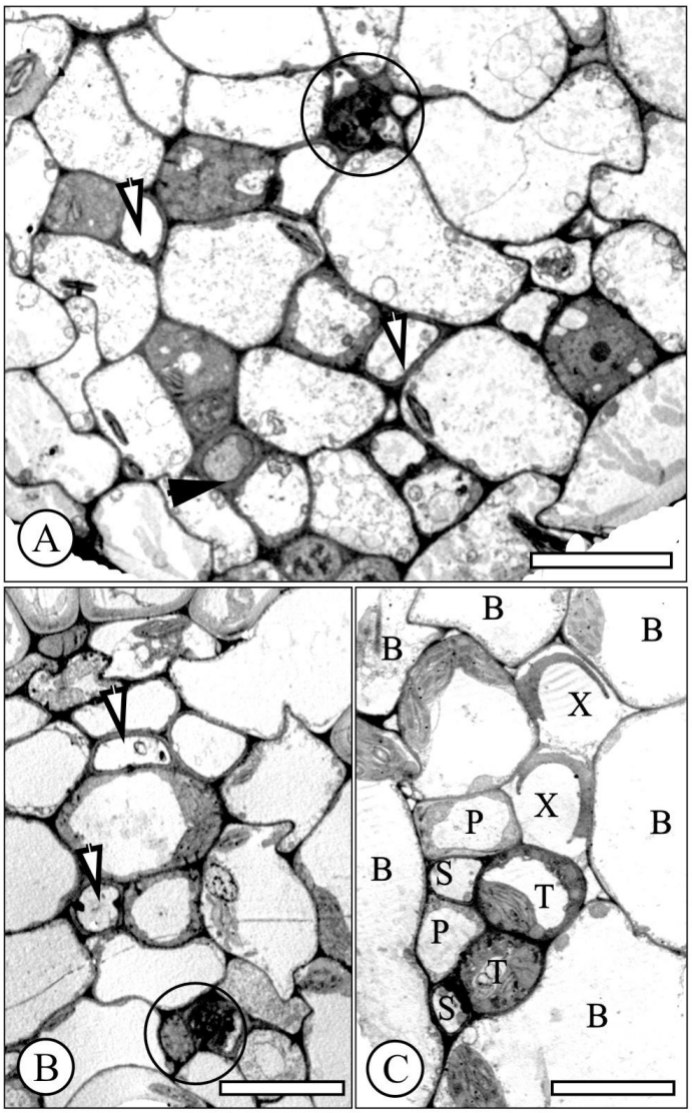

Fig. 5.2: Transmission electron micrographs showing stages of development of minor veins.
(A) shows several minor veins at different stages of development in tissues at LPI 0; mature (open arrowheads) and immature (solid arrowheads) phloem sieve tube are present in the various quaternary veins while the quinternary vein (circled) is still undergoing cell division and contains immature phloem sieve tubes. bar = 1 µm. (B) shows minor veins at different stages of development in developing tissues at LPI 1. Phloem sieve tubes in quaternary veins are mature (open arrowheads) while those in quinternary veins (circled) are at an advanced stage of maturity. bar = 500nm. (C) A mature quinternary vein (LPI 2) containing xylem and phloem cells surrounded by a bundle sheath. The phloem sieve tubes are mature and transfer cells contain wall ingrowths. bar = 2.5 µm. X-xylem; S-phloem sieve tubes, T- transfer cells, P-phloem parenchyma, B-bundle sheath.

sieve tubes in class IV veins are mature (open arrow head) while those in class V veins are at an advanced stage of maturity (circled).
Fig. 5.3 shows phloem tissues at various stages of maturity. In Fig. 5.3 A – B, evidence of nuclear degeneration can be seen in phloem sieve tubes. Figure 5.3 C and D show the development of cell wall protrusions in the transfer cells abutting the differentiating phloem sieve tubes and vascular parenchyma.

Almost, if not all, transfer cells observed had dense cytoplasm which also contained chloroplasts and mitochondria. It was apparent that the wall ingrowths were more frequent in transfer cells of class IV veins than in those of class V veins. The extent of the wall ingrowths in transfer cells observed in each group of minor veins was also largely dependent on the LPI. For example, few, if any wall ingrowths were observed in the transfer cells of class IV veins at LPI 0 (open arrows, Fig. 5.4A). The walls of some transfer cells appear to be in the process of forming ingrowths (closed arrows, Fig. 5.4A). Wall ingrowths appear denser in transfer cells at LPI 0.5 (Fig. 5.4B), and even more so in transfer cells within leaflets at LPI 1 to 2 are even more numerous (Fig. 5.4C). Most of the wall ingrowths in these older leaflets appear long and pronounced.

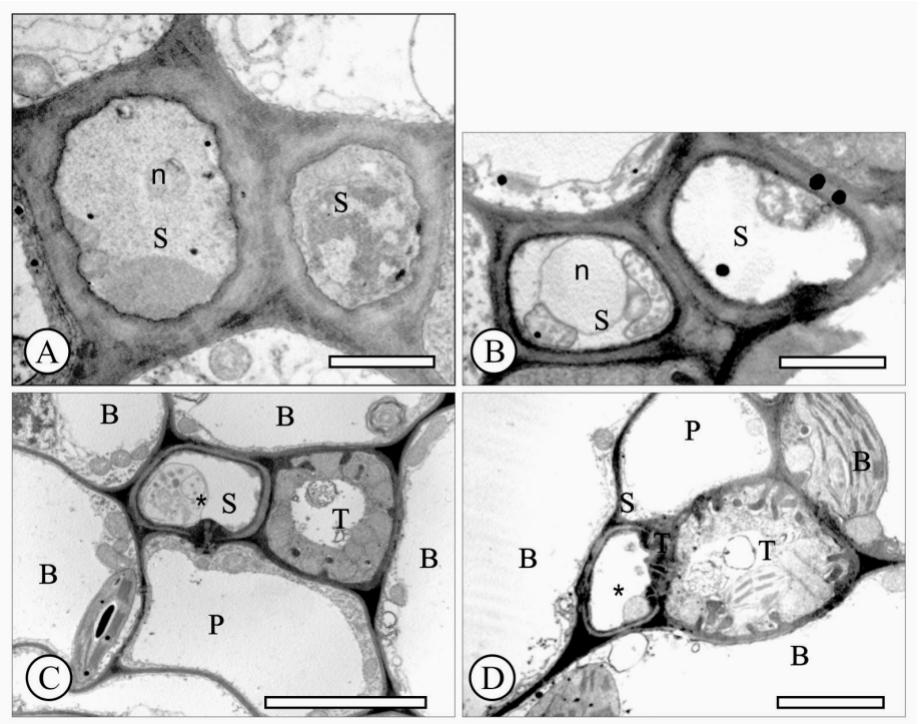

Fig. 5.3 shows phloem tissues at various stages of maturity. Figure 5.3(A) and (B) show nuclear degeneration in the phloem sieve tube. Immature phloem sieve tubes contain protoplast and nucleus (n, A), the nucleus gradually thins out, and the protoplasm is starting to disintegrate (B). (C) and (D) show the development of cell wall protrusions in the transfer cells abutting differentiating phloem sieve tubes and vascular parenchyma. (A) and (B) bar = 1.25 µm; (C) bar = 5 µm; (D) bar = 1 µm. S-phloem sieve tubes, T- transfer cells, P-phloem parenchyma, B-bundle sheath

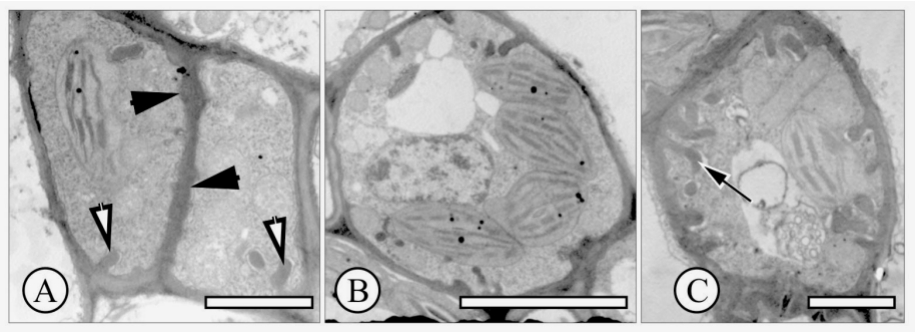

Fig. 5.4: Shows transfer cells in the minor veins of leaflets at different LPI with characteristic wall ingrowths. Very few wall ingrowths (open arrow heads) are apparent in transfer cells at LPI 0 (A) but numerous small projections of wall tissues are evidence of emergent ingrowths and the wall appear to be in the process of producing more wall ingrowths (closed arrow heads). Note the increase in the number of wall ingrowths visible as tissues progressed from LPI 0.5 (B) to LPI 1 (C). Most of the wall ingrowths in leaflets at LPI 1 are exceptionally long, and some form elaborated structures (solid arrow). (A) bar = 1.25 µm; (B) bar = 5 µm; (C) bar = 4 µm

Bent (open arrow heads) and fused (closed arrow head) wall ingrowths became evident in older transfer cells in leaflets of higher LPI.

What was evident was that the structure of the plasmodesmata was dependent on the cell-to-cell interface and not on LPI. The plasmodesmata between the sieve element-transfer cell (SE-TC) complexes were usually single on the phloem sieve tube side and branched on the transfer cell side (Fig. 5.5A) while plasmodesmata at other interfaces were unbranched on both sides, for example between transfer and phloem parenchyma cells (Fig. 5.5B), phloem parenchyma and bundle sheath cells (Fig. 5.5C), phloem parenchyma cells and phloem sieve tubes (Fig. 5.5D), two phloem parenchyma cells (Fig. 5.5E) and bundle sheath and mesophyll cells (Fig. 5.5F). Mature plasmodesmata were present in all tissues from leaflets at LPI 0 to 2, though more aggregates of plasmodesmata were observed in older tissues.

There was no apparent noteworthy difference in the sink/source state or anatomy of plants from different CO_2, nitrogen and nodulation treatment conditions except that plants grown under high [CO_2] accumulated starch at an earlier age (Chapter 6).

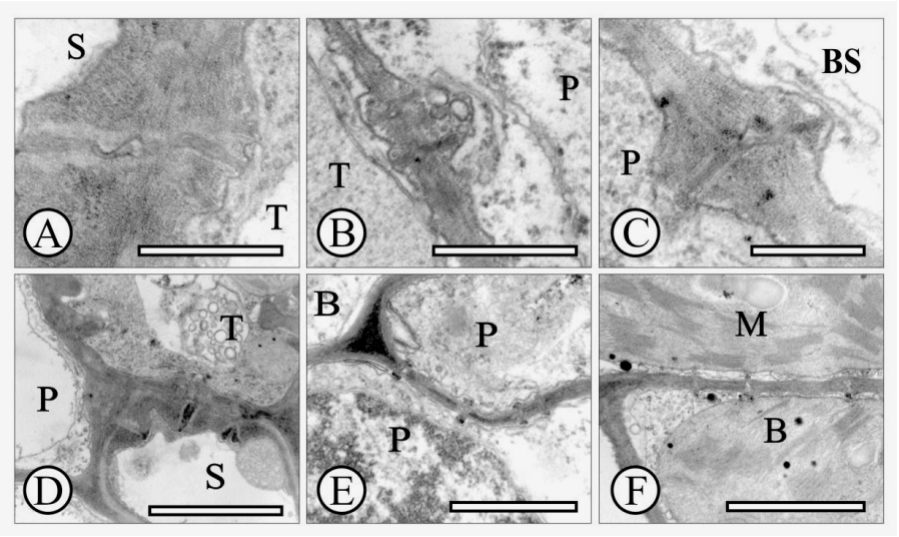

Fig. 5.5: Transverse sections showing plasmodesmata in different cell-to-cell interfaces. (A) Branched pore-plasmodesmata occur between phloem sieve tubes (unbranched) and transfer cells (branched). Unbranched plasmodesmata are present in other interfaces such as between transfer and phloem parenchyma cells (B), phloem parenchyma and bundle sheath cells (C), phloem parenchyma and phloem sieve tubes (D), two phloem parenchyma cells (E), bundle sheath and mesophyll cells (F). (A - C) bar = 3 µm; (D - E) bar = 1.6 µm. SE - phloem sieve tubes, T - transfer cells, PP – phloem parenchyma cells, BS - bundle sheath cells, M - mesophyll cells.

5.3 Discussion

5.3.1 Lamina development

It has been shown that cell division ceases first in the epidermal layers during leaf ontogeny, and that this cessation is followed closely by that of the spongy cells, followed by the palisade (Avery 1933, Esau 1977). Results of the light microscopy study of the lamina of *P. sativum* indicated that cells within leaflets at LPI 0 are compact with few intercellular spaces. Larson and Isebrands (1971) have also shown that in cottonwood, a leaf at LPI 0 is just beginning its phase of rapid expansion and intercellular spaces have not yet been formed between the mesophyll cells. The compact state of the different cellular layers of the lamina at LPI 0 is characteristic of tissues that are at the import stage (Turgeon 1989). The fluorescence experiments in Chapter 4, find support in the studies carried out here.

As mentioned, the appearance of intercellular spaces between the spongy mesophyll cells progresses between LPI 0.5 and 1, and also between the palisade cells at LPI 1. Avery (1933) stated that the continuous enlargement of the epidermal layer after the mesophyll has ceased expanding, results in the pulling apart of the mesophyll cells and hence the formation of intercellular spaces first between the lower mesophyll cells, and later between the palisade cells.

The formation of air spaces in the palisade and spongy mesophyll, which occurred from LPI 0 through LPI 1 in the pea leaflets, was suggested by Fellows and Geiger (1974) as one of the preparatory events which occurs during the transition from sink to source state in developing leaves. The results of the fluorescence experiments which showed that the upper region of the lamina of *P. sativum* undergoes transition from sink to source between LPI 0 and 1, finds support in these studies.

5.3.2 Ultrastructure of the minor vein phloem

The structure of the minor veins of *P. sativum* presented in this chapter supports the findings presented by Wimmers and Turgeon (1991) and is also similar to the data presented for *P. arvense*, as described by Gunning and Pate (1969). The minor veins are collateral, consisting of a variable number of tracheary elements (one to five) with or without associated xylem parenchyma. Sieve tube members are associated with transfer cells and phloem parenchyma. Bundles are enclosed by a chlorenchymatous bundle sheath. Phloem sieve tubes and transfer cells both abut phloem parenchyma and bundle sheath cells. Phloem sieve tubes are always associated with at least one transfer cell. Individual SE-TC complexes are either in direct contact or have phloem parenchyma between them.

5.3.3 Ultrastructure of transfer cells

The transfer cells with their characteristic wall ingrowths is typical of that described by Gunning *et al*. (1968) for transfer cells in minor veins of *P. arvense* leaflets. Transfer cells are sites of known or assumed high solute flux (Wimmers and Turgeon 1991) synonymous with the phloem of the minor veins of source leaves (Gunning and Pate 1969, Bourquin *et al*. 1990), and the development of wall ingrowths has been demonstrated to begin with the onset of solute flux (Gunning and Pate 1969, Folsom and Cass 1986). Two contrasting specializations which are likely to favour efficient absorption of materials into a cell are: (1) enhancement of symplasmic transfer through the development of abundant plasmodesmata and (2) the promotion of capacity uptake from the extracellular environment through increase in the cell's surface:volume ratio. The transfer cells of *P. sativum*, reported in this thesis, like those of their counterpart in *P. arvense* (Gunning *et al*. 1978), are possibly specialized in the latter direction. In the pea plant, the wall ingrowths in transfer cells form part of an irregular layer of wall material deposited secondarily on the primary wall and have loose microfibrillar texture. Wall microfibrils, possibly created by fixation damage, are visible in the space between the plasma membrane and the wall. The plasma membrane appears to have granular or fibrillar material deposited on it and in this is similar to that reported by Gunning *et al*. 1978.

5.3.4 Phloem maturation

5.3.4.1 Transfer cells

The occurrence and protrusion of wall ingrowths in the transfer cells in minor veins increased with LPI up until LPI 1.

5.3.4.1a. LPI 0

The presence of one to two wall ingrowths along with the appearance of new protrusions along the wall of transfer cells of leaflets at LPI 0 is taken as evidence that the emergence of wall ingrowths in transfer cells commences when leaflets are about LPI 0. The onset of the protrusion of wall ingrowths as shown in leaflets at LPI 0 therefore confirms the data from the fluorescence experiments which showed that sink-to-source transition commences at LPI 0 in *P. sativum*.

5.3.4.1b. LPI 1 - 2

Transfer cells of leaflets at LPI 1 had similar distribution of wall ingrowths as did leaflets at LPI 2. It is not surprising that the number of wall ingrowths increase as the leaf matures from LPI 0 to LPI 1, as the wall ingrowths increase in number and surface area as the photosynthetic capacity of the leaflet increases, to ensure an increase in plasmalemma surface area needed for higher assimilate transfer across this transfer cell-sieve tube interface and the subsequent export of the assimilate (Gunning *et al*. 1968). The elaboration of the cell wall ingrowths in transfer cells of leaflets from LPI 1 was also observed by

Wimmers and Turgeon (1991), more especially in minor veins of high-light leaves.

5.3.4.2 Phloem sieve tubes

Mature sieve tube members contain no nucleus or central vacuole but retains a thin layer of peripheral cytoplasm with plastids, mitochondria, and smooth ER (Hoefert 1980; Gagnon and Beebe 1996b).

5.3.4.2a. LPI 0

The class IV veins in lamina sections at LPI 0 contained mature as well as immature phloem sieve tubes, while class V veins appear still to be undergoing cell division. According to Turgeon (1989), leaves at the import phase are still expanding, therefore their minor veins, which are not needed for distribution of imported assimilate, contain immature phloem sieve tubes that are yet to differentiate and undergo further growth to accommodate the expansion within the lamina, before the leaf transits into an exporting state. According to Turgeon and Webb (1976), the phloem sieve tubes mature simultaneously with the companion cells as the leaf ages and import ceases. The gradual progression in phloem sieve tube ontogeny can be seen by looking at phloem sieve tubes in Fig. 53A across to Fig. 5.3D. According to Fellows and Geiger (1974), it appears that the development of mature, relatively open sieve tubes, like phloem loading, is a preparatory step and does not itself trigger the onset of export. Gagnon and Beebe (1996b) have also reported the presence of some mature phloem sieve

tubes along with immature phloem sieve tubes in larger minor veins of importing tissues of *Moricandia arvensis*. Their observations find support in this study, where mature phloem sieve tubes as well as immature phloem sieve tubes were observed in class IV veins of lamina tissues at LPI 0, which have been shown elsewhere (Chapter 4) to be importing. The presence of mature phloem sieve tubes in some minor veins of leaflets LPI 0 therefore supports the argument that the termination of import is in progress at this stage.

5.3.4.2b. LPI 1 - 2

By LPI 1, all phloem sieve tubes in class IV veins appeared to be fully differentiated. Immature phloem sieve tubes were however, apparently still present in some class V veins at LPI 1. This is in agreement with results reported by Turgeon and Webb (1976) who showed that as the leaf of *Cucurbita pepo* L. ages, the minor vein mature progressively from the largest towards the smallest veins. Gagnon and Beebe (1996b) have also reported the presence of mature phloem sieve tubes, usually in large minor veins distal to the import-termination boundary in the apical region of the lamina of *M. arvensis*. In addition, they reported that the smallest minor veins often contained maturing phloem sieve tubes at different developmental stages. Fellows and Geiger (1974) have also shown that a sizable proportion of the population of minor vein phloem sieve tubes in sugar beet (*Beta vulgaris* L.) attained maturity in the older sink region only prior to the initiation of phloem loading.

The presence of mature phloem sieve tubes together with still immature phloem sieve tubes in minor veins of tissues from supposedly exporting lamina regions (see Chapter 4) may show that there is no close correlation between phloem maturation and import termination, as pointed out by Gagnon and Beebe (1996b). However, the increase in the number of mature phloem sieve tubes in young, supposedly importing tissue at LPI 0 to older, supposedly exporting tissues at LPI 2 demonstrates that there is probably an association between phloem maturation and the transition from importing to exporting status as concluded by Gagnon and Beebe (1996b) based on the same reason.

5.3.4.3 Plasmodesmata

Pore-plasmodesmata observed in this study support the report of Wimmers and Turgeon (1991) that all plasmodesmata in *P. sativum*, except those between transfer cells and phloem sieve tubes, are unbranched and that they occur either singly or in aggregates. The plasmodesmata connecting phloem sieve tubes to transfer cells are typical of pore-plasmodesmata between the sieve element-companion cell (SE-CC) complex in other species. Sieve tubes are generally symplasmically connected to companion cells (here, transfer cells) by distinct structures referred to as pore-plasmodesmata units (PPUs) characterized by multiple branches on the companion cell (transfer cell) side and a single branch on the SE side (Ayre *et al*. 2003).

Plasmodesmata were observed in all minor veins between all parenchymatic cells. However, there appears to be no correlation between timing of plasmodesmata development and LPI. Gagnon and Beebe (1996b) have reported that the timing of plasmodesmata development was the same for all minor veins and was associated with SE-CC complex differentiation in *M. arvensis*. The authors reported that specialized plasmodesmata begin to differentiate when SE-CC complexes were still undifferentiated. Specialized plasmodesmata are reported to be structurally mature by the time companion cells appear fully differentiated, although their associated phloem sieve tube members were still immature (Gagnon and Beebe 1996b). Results presented in this chapter support the conclusion of Gagnon and Beebe (1996b) that the lack of correlation between the maturation of specialized plasmodesmata and the arrest of assimilate import seemingly precludes the possibility that the development of the plasmodesmata can be linked to initiation of phloem-loading capacity as argued (see introduction) by Beebe and Evert (1992) and later in Evert *et al*. (1996). The results presented here therefore support Gagnon and Beebe's (1996b) conclusion that the lack of a clear correlation between plasmodesmal development and sink-to-source transition could be a reflection of the secondary role of plasmodesmata in phloem loading.

5.4 Overall conclusion

The data in this chapter support the use of the plastochron index as an accurate measure of the physiological and morphological states of plant organs (Erickson and Michelini 1957, Lamoreaux *et al*. 1978). The plastochron index proved to be a reliable tool in the investigation of the ontogeny of leaflets of *P. sativum*. The results detailed in this chapter clearly showed that some structural changes in the cross section of the lamina and the minor veins are not only correlated with LPI but are also somehow linked to the sink/source state of leaflets. The results presented in this chapter can be used to predict the cross sectional layout of cells in the different layers of the lamina, the presence or absence of immature phloem sieve tubes and the extent of wall ingrowths in transfer cells. Also, the usefulness of the plastochron index as a predictor of developmental state is apparently fully justified.

CHAPTER 6: The Interactive Effects of Elevated CO_2, Nitrogen and Nodulation on the Growth of *Pisum sativum* L.

6.1 Introduction:

Even as recently as the mid 1990s, some researchers proposed that plant growth under high/elevated CO_2 was not affected by environmental stress factors (Idso and Idso 1994), while others have reported or concluded that high [CO_2] effects varied among plant species grown under different environmental conditions (Kimball 1983, Poorter 1993, 1998, Thompson and Woodward 1994, Hunt *et al*. 1995, Ziska *et al*. 1996, Brunce 1998, Wu and Wang 2000). The interactive effects of elevated CO_2 and other environmental factors on plants therefore remain a subject of much discussion. Daepp *et al*. (2001) reported that high growth rate under high nutrient availability promoted a strong response to elevated CO_2. It is well known that legumes with their assumed unlimited access to atmospheric N via symbiotic fixation usually show a stronger response to elevated CO_2 than non-fixing plant species (Zanetti *et al*. 1997). However, other researchers have demonstrated strong positive effects of elevated CO_2 on legumes, relative to effects on non-legume species under conditions which included high mineral nutrient supply (Nijs *et al*. 1989, Körner 1995, Pritchard *et al*. 1997).

Nitrogen availability seems to be fundamental to plant growth under elevated CO_2 conditions. For example, Philips *et al.* (1976) demonstrated that short-term increases in CO_2 levels promoted N_2 reduction in peas (*P. sativum*) by enhancing root nodule functioning, whereas long-term CO_2 enrichment promoted N_2 reduction by increasing plant and root nodule development (due mostly to an increase in root area available for nodule formation) which resulted from excess nitrogen fixed during early period of treatment. However, increased nodulation is not maintained during long-term exposure to elevated CO_2 in the presence of supplemented mineral nitrogen, as further nodule initiation is probably inhibited in such treatments. Rivière-Rolland *et al.* (1996) showed that decrease in Rubisco activity occurs in peas grown with elevated CO_2 coupled to nitrogen-limiting conditions. A threshold value for the N status according to Rivière-Rolland *et al.* (1996) occurs above which Rubisco is not down-regulated by high CO_2.

According to Reddy *et al.* (1998), the effects of increased [CO_2] on growth are primarily due to changes in leaf area production, with smaller effects of increased CO_2 on photosynthetic rate, nitrogen and water use efficiency. Under elevated CO_2, leaf area responses may be limited by deficits in the supply and availability of nutrients or water (Reddy *et al.* 1998). These authors imply that studies on leaf development need to encompass analyses of the rates of leaf

appearance and expansion/elongation which according, to Reddy *et al*. (1993), are important factors that are involved in determining leaf area changes. Given that available leaf area will, under most conditions, regulate the rate of photosynthesis, nutrient cycling and a host of related processes, it is clear that an understanding of the rate of leaf initiation, expansion and maturation under controlled environments becomes essential, if we are to fully comprehend the overall effects of sustained elevated CO_2 on growth and productivity, especially of important staple crop plants such as the legumes.

It is for this reason that the application of plastochron in the study of the overall effects of [CO_2] on the growth of *P. sativum* was considered to be vital to our understanding of the effects of elevated CO_2 on the appearance and rate of growth of leaves. Periodic leaf length measurements used in plastochron index analysis were used in determining leaf elongation rate, while plastochron duration is equal to the rate of leaf appearance (Ade-Ademilua and Botha 2005). Thus, plastochron index analyses would provide data needed for the complete study of leaf development under elevated CO_2.

The results of experiments aimed at the determination of the influence of [CO_2], N availability, and nodulation on the rate of appearance and elongation of leaves of *P. sativum* are reported here. They demonstrate that use of the plastochron index and plastochron duration are excellent

measures for the determination of relative plant growth rate on a per unit time basis, under experimental conditions.

6.2 Results

In order to gain a clearer understanding of some of the effects of elevated CO_2 on key growth parameters in the pea plant, the subsequent study was undertaken under fixed and controlled elevated CO_2 conditions and the data obtained under these conditions were compared to experiments conducted under ambient CO_2 under controlled environment conditions. Elevated (E) CO_2 (1000 µmol mol^{-1}) and ambient (A) CO_2 (380 µmol mol^{-1}) were studied in plants grown under the following conditions:

Nodulated plants (R) supplied with nitrogen free-nutrient solutions (n)

Nodulated plants (R) supplied with nutrient solution containing mineral nitrogen (N) and, Non-nodulated plants (r) supplied with nutrient solution containing mineral nitrogen (N).

Under controlled environment, seeds normally germinated within 2 days after sowing in all treatments. Leaflet elongation was rapid (exponential phase) for the first five days after leaf unfolding. The rate of increase in leaflet elongation was therefore determined using values within the period of exponential growth, that is, five days.

6.2.1 Effect of elevated CO_2 on nodulating plants without mineral N supply

The average plastochron index (PI) of nodulated plants supplied with N-free nutrient solution grown under elevated CO_2 (REn) was significantly higher than that of their counterparts under ambient CO_2 (RAn), throughout the experimental period. This is illustrated in Fig. 6.1A ($p=0.01$ at 11 DAG and $p=0.001$ from 12 to 20 DAG). The plastochron duration (inverse of slope) of REn-treated plants was shorter (2.64 ± 0.06days) than that of RAn-treated plants (2.74 ± 0.12days) but the data were not significantly different at $p=0.05$. This suggests that a positive response to elevated CO_2 by nodulated plants occurs during the very early stages of growth and that these plants were advantaged (in terms of plastochron age) over their counterparts grown under ambient CO_2. Figure 6.1B shows that leaflets of REn-treated plants grew faster (3.58 ± 0.39mm day^{-1}) than those of RAn plants (3.01 ± 0.16), but the difference was also not significant at $p=0.05$. the statistical analysis of the data suggest that long-term exposure of nodulated *P. sativum* L to elevated CO_2 does not seem to influence the growth of leaves positively.

6.2.2 Effect of elevated CO_2 on nodulating plants under mineral N supply

Figure. 6.2A shows the rates of change in average PI of plants over time. With the exception of 19 and 20 DAG, the average PI of

nodulating plants supplied with N+ nutrient solution under elevated CO_2 (REN) was significantly higher (p=0.05) than that of plants grown under ambient CO_2 (RAN) throughout the experimental period. Whilst the average plastochron duration was 2.30 ± 0.07days under elevated CO_2 (REN) and 2.34 ± 0.07 under ambient CO_2 (RAN), the values were not significantly different at p=0.05 as was the case in nodulating plants treated with N- nutrient solution,. A positive response in growth under short term exposure to elevated CO_2 is also evident. However, unlike the REn and RAn-treated plants, Fig. 6.2B shows that leaflet elongation rate (LfER) was higher (p=0.05) in REN-treated plants (5.70 ± 0.27) than in RAN--treated plants (4.67 ± 018) and the values were significantly different at p=0.05. This indicates therefore, that LfER is enhanced by elevated CO_2 under high nitrogen supply (fixed nitrogen and mineral nitrogen). Curiously, lateral branches emerged from node 0 in plants under REN treatment shortly before flowering of the mother plants and were morphologically similar to the mother plant which bore them, but they did not produce flowers. Lateral branches emerged in two plants under RAN treatment after the other plant had produced pods but subtended by miniature stalks with minute leaves.

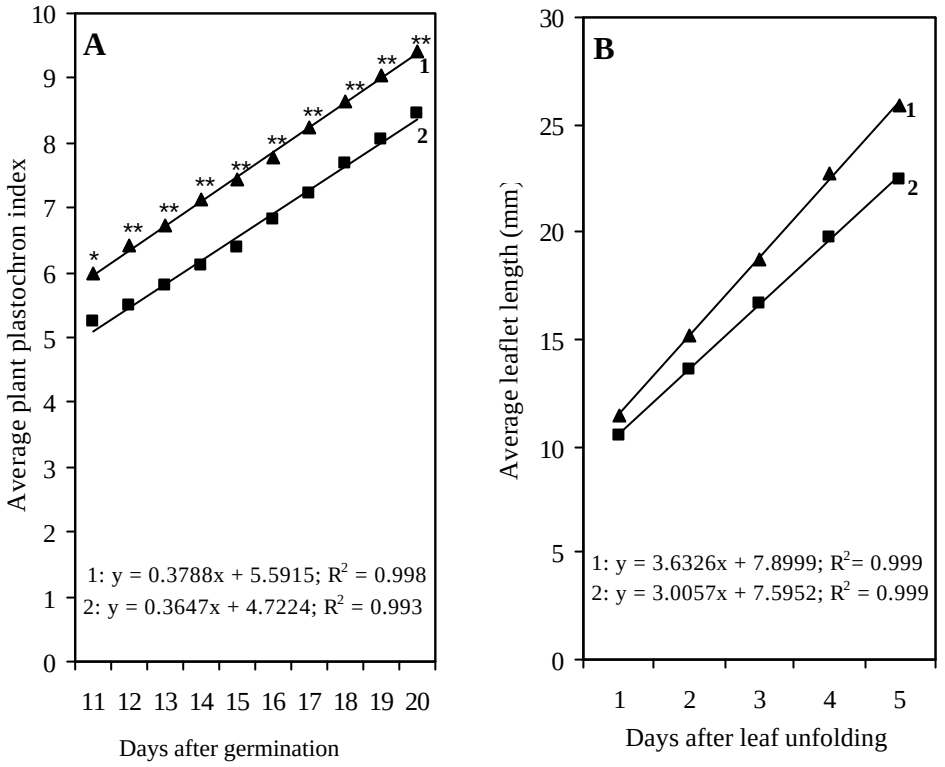

Fig. 6.1 : Change in average plant plastochron index (A) and length of leaflets on node 6 (B) with time (with slope embedded), of nodulating plants supplied with N+ nutrient solution under elevated (REn,1) and ambient (RAn, 2) CO2. Values at points with * or ** are significantly higher than those on similar X point at p=0.01 or p=0.001 respectively.

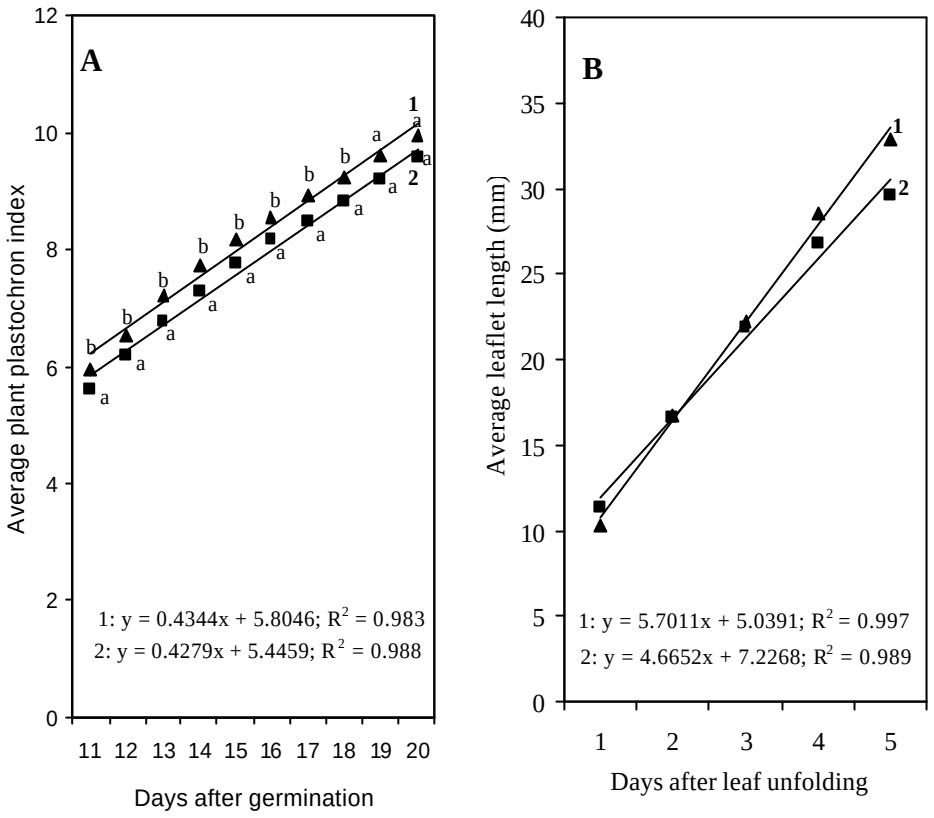

Fig. 6.2: Change in average plant plastochron index (A) and length of leaflets on node 6 (B) with time (with slope embedded), of nodulating plants supplied with N+ nutrient solution under elevated (REN,1) and ambient (RAN, 2) CO2. Values on same X point bearing same letter are not significantly different at p=0.05.

6.2.3 Effect of elevated CO_2 on non-nodulating plants under mineral N supply

The rate of change in average PI of non-nodulating plants supplied with N+ nutrient solution under elevated CO_2 (rEN) and ambient CO_2 (rAN) is shown in Fig. 6.3A. The plastochron duration in rEN-treated plants was significantly higher at p=0.05 (2.03 ± 0.05 days) than that calculated for rAN plants under (2.21 ± 0.06 days). PI values throughout the experimental period were significantly higher at p = 0.001 in rEN-treated plants than in rAN-treated plants. Similarly, the leaflets elongation rate (Fig. 6.3B) was significantly higher at p=0.01 in rEN-treated plants (5.87 ± 0.18) than in rAN plants (4.72 ± 0.36). The data indicate that both short-term and long-term exposure to elevated CO_2 enhanced leaf growth in non-nodulating *P. sativum* plants, which were supplemented with mineral N. The emergence of lateral branches initiated at node 2 was somewhat curious as, unlike those grown in the nodulating plants supplied with mineral N, which emerged during the reproductive stage, branches emerged from node zero of rEN-treated plants during vegetative stage (17 DAG; Fig. 6.4). These branches produced flowers and then fruits, almost at the same time as the mother plants though on lower nodes in rAN-treated plants,

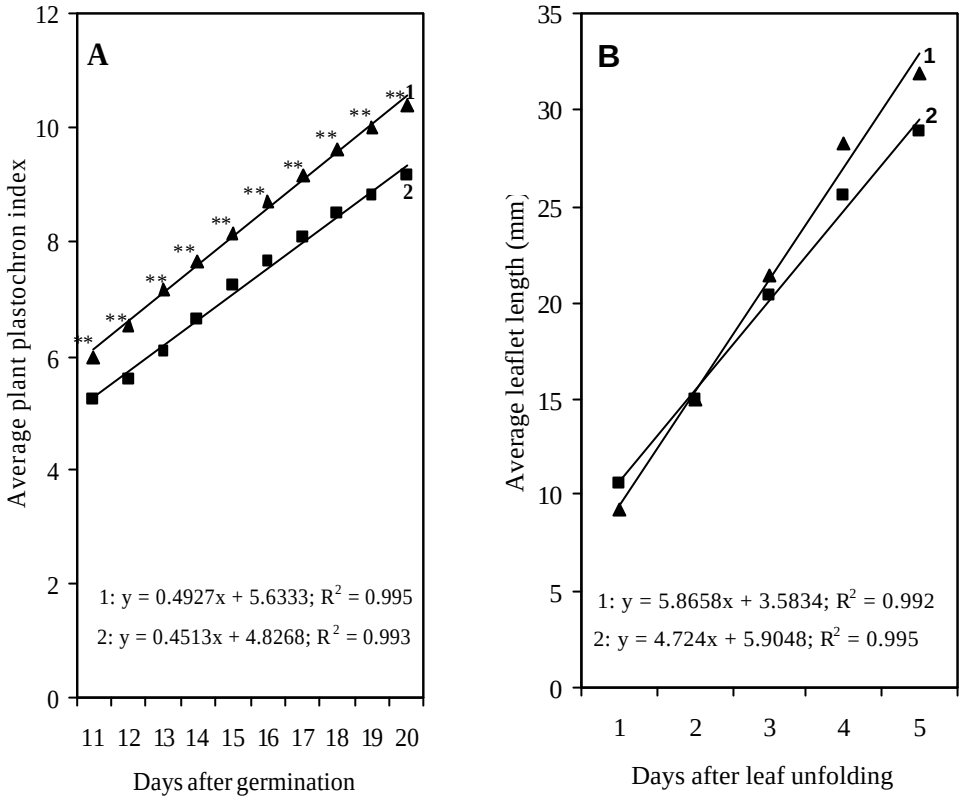

Fig. 6.3: Change in average plant plastochron index (A) and length of leaflets on node 6 (B) with time (with slope embedded), of non-nodulating plants supplied with N+ nutrient solution under elevated (rEN,1) and ambient (rAN, 2) CO2. Values at points with ** are significantly higher than those on similar X point at p=0.001.

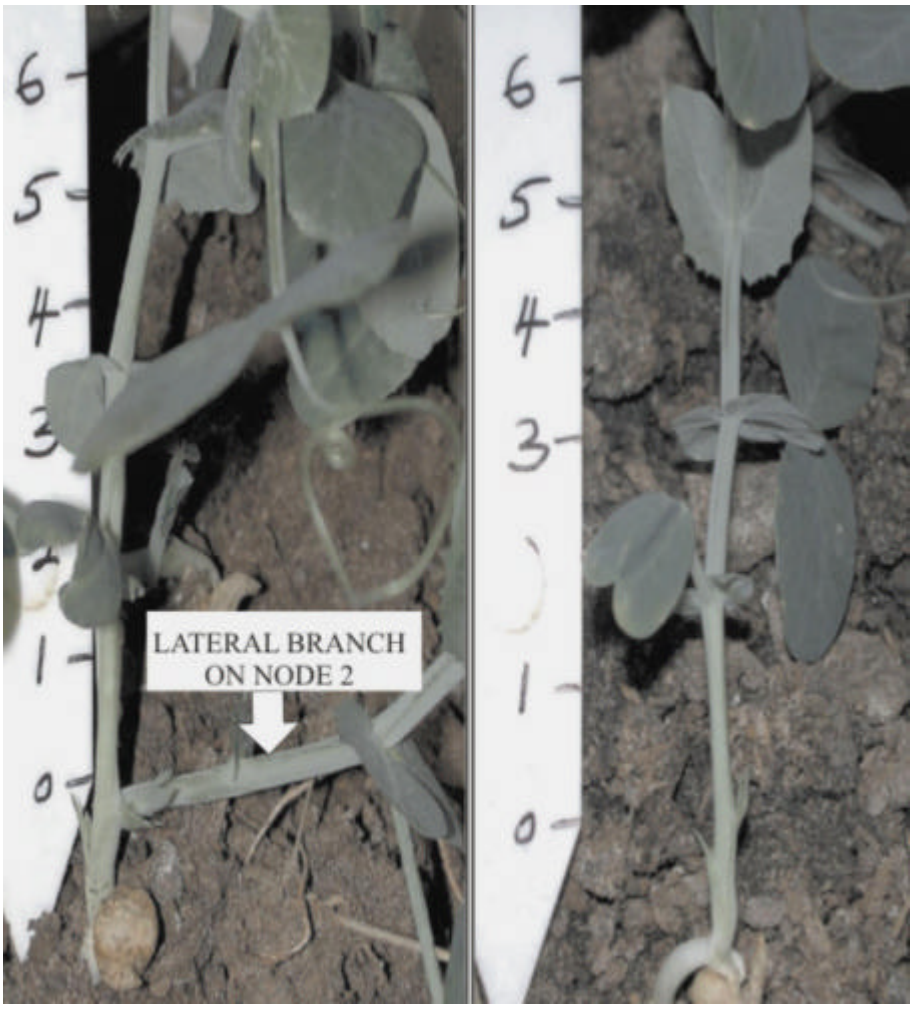

Fig. 6.4: Basal portion of non-nodulating plants supplied with mineral N show (A, left) lateral branch on node 2 under elevated CO2 (rEN); (B, right) no lateral branch on node 2 under ambient CO2 (rAN).

in contrast to those in nodulating plants supplied with mineral N that did not flower. Branches were only noticed on three plants under rAN treatment, and they emerged after the mother plants had reached pod-filling stage, and grew morphologically like the mother plant but did not produce flowers.

6.2.4 Interactive effect of elevated CO_2, nodulation and mineral N supply on rate of leaf appearance (RLA)

The average plastochron indices of all plants grown under elevated CO_2 were significantly higher than that of their counterparts grown under ambient CO_2 throughout the period of analysis, as evident in the significant differences between data of plants under elevated CO_2 as against those of plants under ambient CO_2 in Figs. 6.1, 6.2 and 6.3. The average PI of nodulating plants supplied with N+ nutrient solution under elevated CO_2 (REN) was significantly higher ($p=0.05$) than that of plants under ambient CO_2 (RAN) throughout the experimental period, except for 19 and 20 DAG.

Table 6.1 shows a matrix comparison (horizontal against vertical) of the rate of leaf appearance (plastochron duration) in plants under all treatments. A comparison of RAn and RAN-treated plants shows that the supply of mineral N to nodulating plants under ambient CO_2 increased RLA (plastochron duration) significantly at $p=0.05$. However, significant induction of RLA due to mineral N supply to nodulating plants (REn versus REN) did not take place under elevated CO_2. Elevated CO_2 also had no effect on the RLA of nodulated plants (compare RAn to REn, RAN to REN). RLA in plants grown under ambient CO_2 was significantly higher in plants in which N was supplemented (rAN), than under fixed N alone (RAn), however, there was no significant difference between nodulated and non-nodulated

plants, when nodulated plants were supplied with additional mineral N (RAN versus rAN). However, elevated CO_2 induced RLA significantly in non-nodulated plants above that of nodulated plants which were grown with or without mineral N supplement (REn and REN versus rEN).

With RAN serving as control, Fig. 6.4 further illustrates the interactive effects of elevated CO_2, nodulation and mineral N on RLA, based on the percentage increase in RLA in other treatments, compared to RAn-grown plants. The increase in RLA under ambient CO_2 due to mineral N supply in both nodulating (RAN, 17%) and non-nodulating (rAN, 24%) plants respectively, was significant at $p=0.05$ (Table 6.1). Increase in RLA (4%) due to elevated CO_2 was not significant in nodulating plants without mineral N supply (REn). The further increase in RLA (2%) in nodulating plants above that already induced by mineral N supply (REN) was also not significant. Figure 6.4 therefore illustrates that RLA was only significantly affected by mineral N supply in nodulating plants. Furthermore, the effect of elevated CO_2 was only significant in non-nodulating plants supplied with mineral N (rEN).

Table 6.1: Matrix comparison of differences between plastochron duration (rate of leaf appearance, RLA) of P. sativum under all treatments.

Nodulating plants supplied with N-nutrient solution under elevated (REn) and ambient (RAn) CO2; nodulating plants supplied with N+ nutrient solution under elevated (REN) and ambient (RAN) CO2; and non-nodulating plants supplied with N+ nutrient solution under elevated (rEN) and ambient (rAN) CO2.

	Nodulating				Non-nodulating	
	No mineral N supply		With mineral N supply			
	Elevated (REn)	Ambient (RAn)	Elevated (REN)	Ambient (RAN)	Elevated (rEN)	Ambient (rAN)
REn						
RAn						
REN		b				
RAN	b	b				
rEN	**	**	*	*		
rAN	b	b			b	

Note: blank cells show no significant difference; b indicates significant difference at p=0.05; * indicates significant difference at p=0.01; ** indicates significant difference at p=0.001.

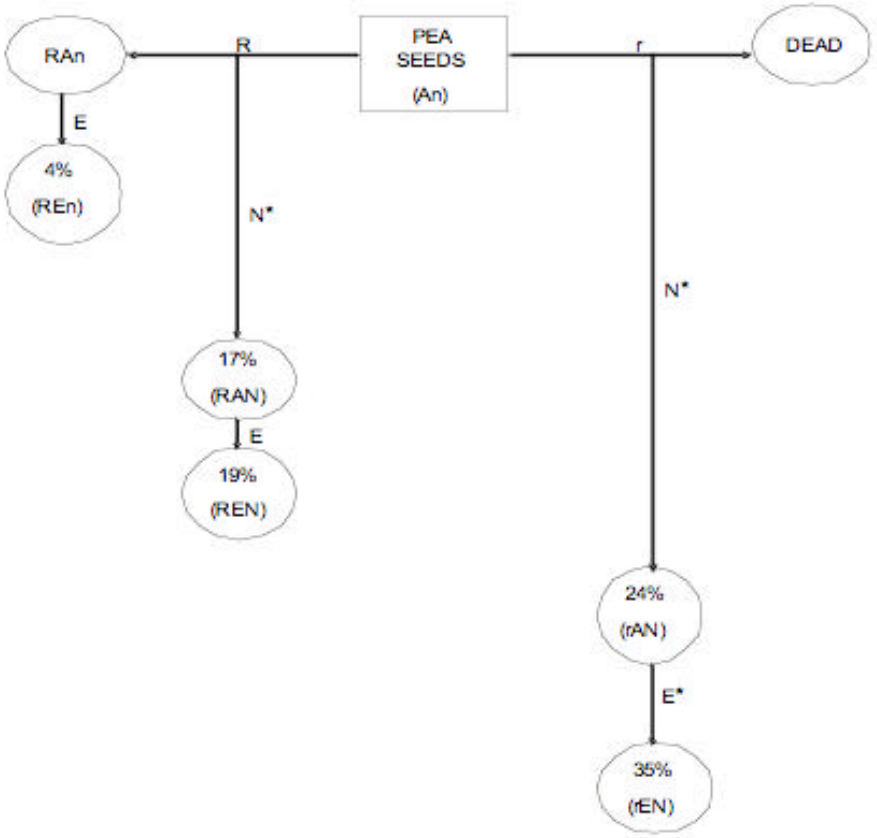

Fig. 6.4: Summary of the interactive effects of nodulation, elevated CO2 and N status on the rate of leaf appearance (RLA, plastochron duration) in plants under the different treatment conditions compared to that of nodulating plants under ambient CO2 without N supply (RAn) as presented in Ade-Ademilua and Botha (2004). * shows that the treatment had a significant effect. (R - nodulation/Rhizobium, r - absence of nodulation, N - mineral N supply, n - no mineral N supply, A - Ambient CO2, E - elevated CO2).

6.2.5 Interactive effect of elevated CO_2, nodulation and mineral N supply on leaflet elongation rate (LfER)

The matrix comparison (horizontal against vertical) of the leaflet elongation rate (LfER) between treatments in Table 6.2 shows some interesting results. Mineral N enhanced the LfER of nodulated plants irrespective of CO_2 condition (compare RAn to RAN and REn to REN). Elevated CO_2 only enhanced LfER in nodulated plants in the presence of mineral N supply. LfER was significantly higher in non-nodulated than in nodulated plants (RAn against rAN). The difference becomes insignificant with the addition of mineral N to nodulating plants (RAN compared to rAN). Similar results were obtained under elevated CO_2 (compare REn to REN, and both to rEN).

The interactive effects of elevated CO_2, nodulation and mineral N on LfER, based on the percentage increase in RLA in other treatments compared to RAn, is further illustrated in Fig. 6.5. Mineral N supply increased LfER significantly in both nodulating (RAN, 55%) and non-nodulating (rAN, 57%) plants. However, the effect of elevated CO_2 on LfER was only significant where mineral N was supplied to nodulating plants (REN, 90%) and even more significant in non-nodulating plants supplied with mineral N (rEN, 97%).

Table 6.2: Matrix comparison of differences between leaflet elongation rate (LfER) of *P. sativum* under all treatments. Nodulating plants supplied with N- nutrient solution under elevated (REn) and ambient (RAn) CO2; nodulating plants supplied with N+ nutrient solution under elevated (REN) and ambient (RAN) CO2; and non-nodulating plants supplied with N+ nutrients solution under elevated (rEN) and ambient (rAN) CO2.

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	Nodulating				Non-nodulating	
	No mineral N supply		With mineral N supply			
	Elevated (REn)	Ambient (RAn)	Elevated (REN)	Ambient (RAN)	Elevated (rEN)	Ambient (rAN)
REn						
RAn	**	**				
REN	b	**	b			
RAN	**	**		**		
rEN	b	**	b		*	
rAN						

Note: blank cells show no significant difference; b indicates significant difference at p=0.05; * indicates significant difference at p=0.01; ** indicates significant difference at p=0.001.

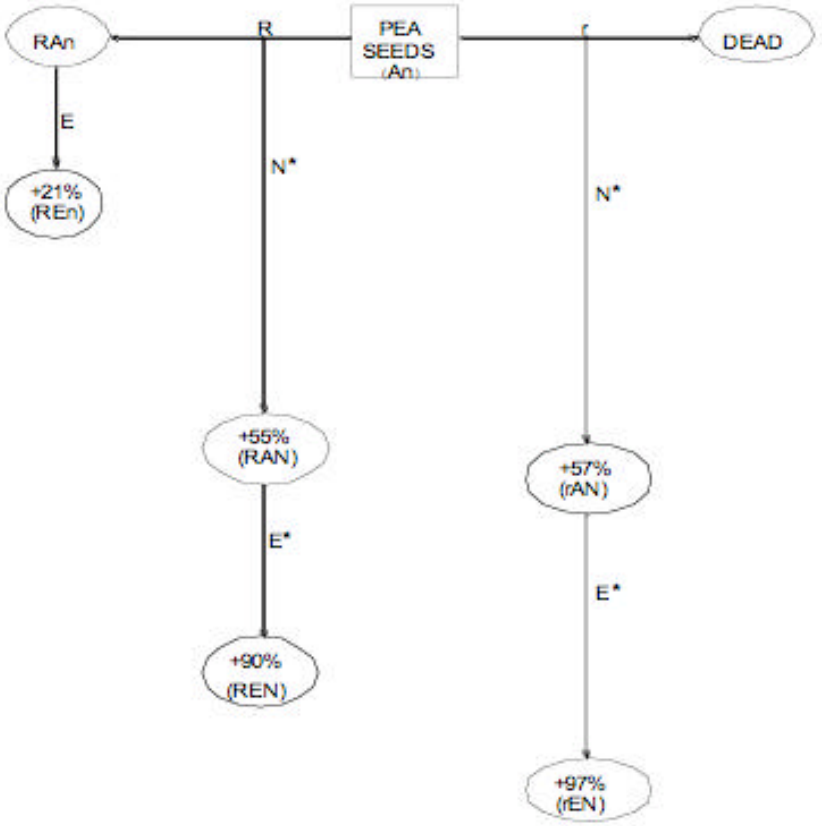


Fig. 6.5: Summary of the interactive effects of nodulation, elevated CO2 and N status on leaflet elongation rate (LfER) in plants under the different treatment conditions compared to that of nodulating plants under ambient CO2 without N supply (RAn) as presented in Ade-Ademilua and Botha (2004).
* shows that the treatment had a significant effect. (R - nodulation/Rhizobium, r - absence of nodulation, N - mineral N supply, n - no mineral N supply, A - Ambient CO2, E - elevated CO2).

6.2.6 Branching under elevated CO_2

Branching was predominant under elevated CO_2 in the presence of mineral N supply. Under elevated CO_2, non-nodulating plants produced branches during vegetative stage (PI>7), in contrast to nodulating plants which produced branches only at flowering stage (PI>15). However, only branches produced by non-nodulating plants flowered though at a lower node than the mother plants. The few plants that produced branches under ambient CO_2 did so at post-fruiting stage. Interestingly, only branches from non-nodulating plants (rAN) had properly formed leaves, those from nodulating plants had very minute leaves.

6.2.7 Increase in starch grains under elevated CO_2

Fig. 6.6 shows starch grains in the mesophyll chloroplasts of leaflets at LPI 0.5 under elevated (A) and ambient (B) CO_2 conditions. Starch grains in the chloroplasts of leaflets under elevated CO_2 appear to be more numerous than those present in the chloroplasts of leaflets at same LPI under ambient CO_2.

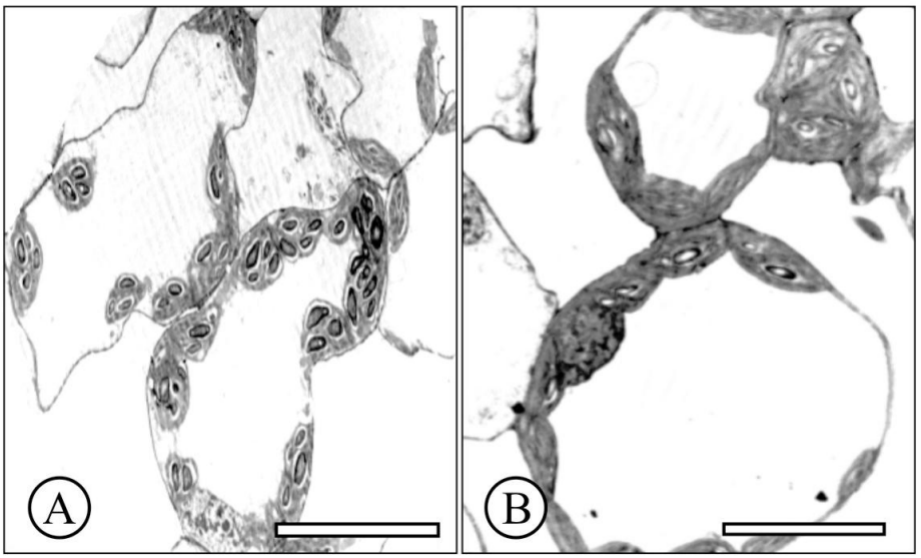


Fig. 6.6: Transmission electron micrographs of typical mesophyll cells in the lamina at LPI 0.5 in plants under elevated (A) and ambient (B) CO2 conditions. Plants under elevated CO2 accumulated more starch grains in the chloroplast than plants under ambient CO2 conditions.

6.3 Discussion

C_3 species are known to be able to utilize elevated [CO_2] to support faster growth, especially during the early stages of growth (Murray 1995). The insignificant differences in plastochron duration values of plants grown under elevated CO_2 from 11 DAG show that short-term exposure to elevated CO_2 enhanced the growth of *P. sativum* plants by inducing an enhanced plastochron index (PI) rate. However under elevated CO_2 conditions, enhanced plant growth occurred only in non-nodulated plants in a long-term experiment in which nitrogen was supplied.

6.3.1 Effects of elevated CO_2, nodulation and mineral N supply on general plant growth in terms of rate of leaf appearance.

The rate of leaf appearance (RLA) was significantly increased in the absence of nodules with mineral N supply. Mineral N supply increased RLA significantly under ambient CO_2. Elevated CO_2 only affected RLA significantly (positively) in plants which did not have nodules. The observations thus support the contention that nutrient availability, especially nitrogen, affects RLA (Truong and Duthion 1993, Turc and Lecoeur 1997). The influence was however greatly reduced under elevated CO_2, suggesting a lack of interaction between elevated CO_2 and mineral N in the influence of RLA. Field experiments using *Lolium perenne* and *Trifolium repens* in managed

grassland have established that the response of vegetative growth to elevated CO_2 in terms of nitrogen availability is nitrogen-dependent in grasses but independent in nitrogen-fixing legumes (Hebeisen *et al*. 1997, Daepp *et al*. 2000, Wagner *et al*. 2001). Proportional allocation of photosynthates is another factor which may prevent the effect of sustained elevated CO_2 being established significantly in plants with access to both fixed nitrogen and mineral nitrogen (Kutík *et al*. 1995). Results of the experiments reported in this chapter suggest that these statements could be true for RLA in nodulating *P. sativum* plants.

The temperature range in all growth conditions used in the experiments was maintained at identical levels under controlled environmental conditions. According to Turc and Lecoeur (1997), RLA is solely dependent on temperature; therefore its value should not be significantly different in plants under the same CO_2 conditions. However, the significant increase in RLA in non-nodulated plants under elevated CO_2, points to a possibility of an increase in the production of photosynthates which, unlike in the case of nodulated plants where assimilates are being shared by the plant as well as by the nodules, the non-nodulated plants can make full use of these resources. As a result, non-nodulated plants have a larger carbohydrate pool available to boost RLA compared with nodulated plants grown under elevated CO_2 conditions. The results support those of Truong and Duthion (1993), which showed that dry matter accounts for variability

in RLA in peas under same temperature conditions. Interestingly, an increase in photosynthesis in non-nodulating *P. sativum* grown under 1000 µmol mol^{-1} and high N supply has been reported by Riviere-Rolland *et al.* (1996). Philips *et al.* (1976) showed that long-term exposure to very high (1200 µmol mol^{-1}) CO_2 with additional mineral nitrogen supply resulted in vigorous, balanced growth of pea plants with no detrimental effect on nitrogenase activity. A major flaw in the experimental procedure was that Philips *et al*. (1976) observations were based on 6 h per day exposure of peas to elevated CO_2 - this was hardly "continuous" exposure! Continuous exposure to elevated CO_2, as in the case of the experiments currently being reported in this chapter, possibly inhibited nodule activity completely in nodulated plants which were supplied continuously with mineral N, thus fixed nitrogen perhaps did not contribute positively to the growth of the leaves of plants under elevated CO_2.

6.3.2 Effects of elevated CO_2, nodulation and mineral N supply on leaflet elongation rate (LfER)

It is clear that high growth rate under high nutrient availability promotes a strong response to elevated CO_2 (Daepp *et al*. 2001). Various experiments have demonstrated that the strong growth effects of elevated CO_2 on legumes, relative to effects seen in non-legume species, are obtained only in the presence of high mineral nutrient supply (Nijs *et al*. 1989, Körner 1995, Pritchard *et al*. 1997). In the

experiments reported in this chapter, elevated CO_2 enhanced LfER significantly in plants supplied with mineral N, irrespective of the presence or absence of nodules. LfER was enhanced significantly by mineral N supply in nodulating plants under both ambient and elevated CO_2 conditions. Clearly, absence or presence of nodules does not appear to affect LfER in plants supplied with mineral N.

6.3.3 Branching

The formation of branches in non-nodulating and nodulating plants supplied with N which were seen in the experiments described in this chapter support the concept of a reduction in apical dominance in the plants under elevated CO_2. Experiments have shown that decapitation of apical bud in *P. sativum* L results in growth of lateral buds on lower nodes (Stafstrom 1995). This has been linked to a reduction in the lateral bud growth inhibitor indole-3-aldehyde in peas with decapitated or aborted shoot tips (Nakajima *et al*. 2002). Branch shoot morphology in *P. sativum* was influenced by the nodal position of the bud from which it was derived, the developmental stage of the plant at the time when the bud was stimulated to grow, hormonal signals from the plant and nutrient availability (Stafstrom *et al*. 1993). This explains the difference in the morphology of branches noticed under the different treatments in this experiment, and could account for the differences in the growth of lateral branches which emerged on plants under elevated CO_2 during the vegetative stage to those which

appeared at the flowering stage in nodulating plants grown under elevated CO_2 and, even more in those that appeared at fruiting stage in plants grown under ambient CO_2. The difference between branches that appear at the fruiting stage in both non-nodulating plants and nodulating plants under ambient CO_2 may be due to the availability of nutrients (nitrogen) - more so in non-nodulating plants than in nodulating plants - and this reason could also apply to plants under elevated CO_2. It is not surprising that branches on non-nodulating plants grown under elevated CO_2 produced flowers at nodes lower than the mother plants, experiments have shown that the node of floral initiation of branch shoots differ from that of the main shoot in the pea plant. The morphology of the main shoot and branch shoot are different with regard to the number of primodia initiated by their apical meristems prior to the onset of reproductive development (Stafstrom and Sussex 1988, 1992; Stafstrom *et al*. 1993, Stafstrom 1995). Lateral branching may also be linked to changes in biomass allocation and have been reported to occur in loblolly pine seedlings under elevated CO_2 (Tissue *et al.* 1997), and Stitt and Krapp (1999) have proposed an involvement of nitrogen supply. Nitrogen fixation and nodulation might affect yield by altering C allocation within the host, especially to the stem, as nitrogen-fixing root nodules are themselves strong carbon sinks and potentially large amounts of carbon may be diverted away from lower priority sinks such as the stem (Stitt and Krapp 1999). Alternatively, nitrogen fixing plants may compensate by

fixing more C, or fixing it at higher rate (Paul and Kucey 1981), thereby increasing the total amount of C available to fill sinks. This should enable lower priority sinks to be filled sooner in nodulating than they would be in non-fixing plants (Arnone and Gordon 1990). The results of these experiments show that in *P. sativum* there was greater likelihood of allocation of dry matter more to nodules than to the lower priority sink like the stem in nodulating plants, while the absence of nodules in non-nodulating plants promoted allocation of excess dry mass to the stem. Mineral N supply to nodulating plants definitely aided the allocation of dry mass to the stem after the mother plant had flowered but at a lower rate compared to non-nodulating plants.

6.3.3 Starch grains

Anatomical results showed that leaflets under elevated CO_2 appear to accumulate more starch grains in their chloroplasts than leaflets at same LPI under ambient CO_2. Increase in the accumulation of starch grains in chloroplasts under elevated CO_2 have been widely reported (Vu *et al*. 1989, Mjwara 1996, Woodward *et al*. 1991, Arp 1991, Utriainen and Holopainen 1998; Teskey 1995) and according to Arp (1991), this may be associated with down regulation of photosynthesis under long-term exposure to high CO_2 conditions. No other significant differences in anatomical features of leaflets at same LPI under different growth conditions were observed.

The data presented here demonstrate conclusively that whereas chronological age is a unit representing a time scale, plastochron index serves as a measure of the rate of growth. The data reported here show that several interpretations can be extrapolated by using PI in growth studies, thereby not only reducing the number of plants required for sampling but also experimental cost and sampling error.

CHAPTER 7: General Discussion and Conclusion

The experiments that are reported in this thesis support the use of the plastochron index (PI) as a morphological time scale and numerical index that represents and reflects the developmental status of the pea plant. The index was found to be a useful tool in quantitative and qualitative work involving analysis and prediction of plant growth and development, thus confirming Erickson and Michelini (1957), Larson and Isebrands (1971) and Lamoreaux *et al*.'s (1978) reports. This research involved the investigation of various morphological and physiological events that together, I believe, serve as a template for the prediction of the growth and development of, and reactions to different growth conditions by *Pisum sativum* L. This was undertaken because of the statements by Larson and Isebrands (1971) suggesting that PI could well be of major use as a predictor of plant growth.

7.1 Determination of PI

In Chapter 3, I discuss the many inherent problems associated with previously used parameters in the determination PI in *P. sativum*. What was centrally important in this research was to stay within the constraints proposed originally by Erickson and Michelini in 1957. Leaflet length met the criteria proposed by Erickson and Michelini (1957) and Lamoreaux *et al.* (1978) as a suitable parameter for calculating plastochron index, as early leaflet growth in peas occurred

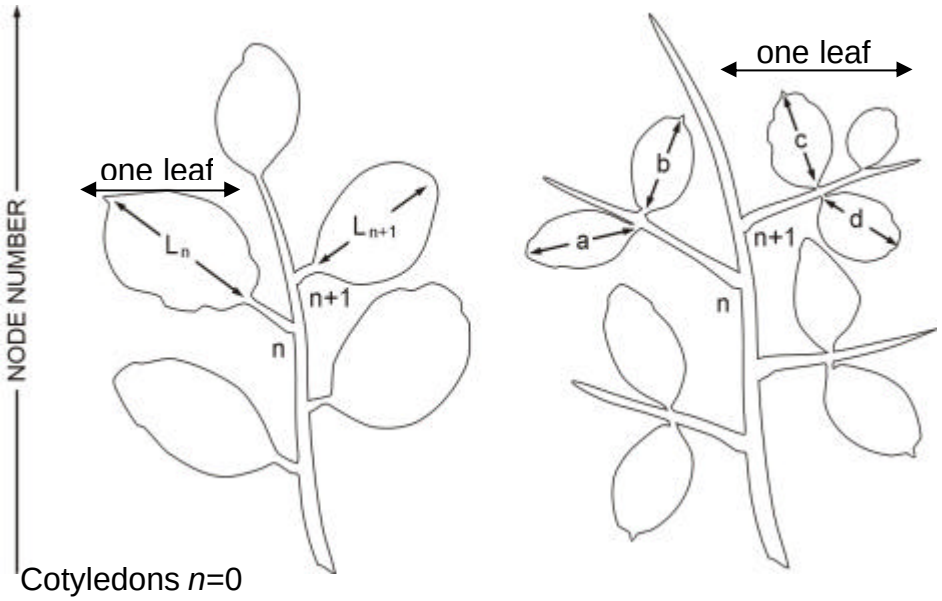
exponentially and, secondly, the early stages of growth of successive pairs of leaflets occurred at the same relative growth rate. The use of leaflet growth was thus shown to be a statistically reliable measure of plastochron index in peas. More importantly, the method is shown to be non-destructive and was thus in agreement with Larson and Isebrands' (1971) requirement. The results of the PI analysis carried out using the formula (Equation 2, Chapter 3) confirmed that plant age can best be expressed using the plastochron index, which is a precise measure of the time interval between the initiations of successive events – in the case of peas, of successive pairs of leaflets.

The data presented in this thesis strongly support the use of average leaflet length in calculating PI. As such, this can be extended to other species with compound leaves (as in the case of *P. sativum* used here). The reliability of the PI formula was confirmed using the long-stemmed Black-eyed Susan pea variety (Ade-Ademilua and Botha 2005). The results proved that the PI formula is appropriate for short-stemmed pea varieties like Greenfeast variety used in this thesis, but that it was applicable to long-stemmed varieties also. In his 1976 paper, Erickson defined the plastochron index in decussate-leaved plants as "the interval between initiations of successive pairs of leaves". The definition of PI in compound-leaved plants would therefore be "the interval between initiations of leaflets on successive leaves". The first pair of leaflets (basal) in compound pea leaves was

used as those which are produced towards the tip of the leaf were found to vary with node position of the compound leaf on the plant. The difference in the approach needed when measuring leaflet length in plants with compound leaves such as that of peas, compared to simple-leaved plants, is illustrated in Fig. 7.1. In the simple-leaved plant A, the length of a leaf (*n*) that is longer or equal to ?, and that of its succeeding leaf (*n*+1) whose length is less than the pre-determined reference length ?, are used in calculating PI. In the compound-leaved plant B, Pi is calculated using the average length of leaflets a and b of leaf (*n*), which must be longer or equal to ?, and that of leaflets c and d on the succeeding younger leaf (*n*+1), which has leaflet lengths that are less than ?. It is important to stress that the basal (first pairs) leaflets are measured (see leaf *n*+1 of plant B, Fig. 7.1) in each case. It should be noted that these basal leaflets assume the plastochron age of the leaf on which they are borne and leaf plastochron index remains as derived by Erickson and Michelini (1957) . as

$$PI \; ? \; n \; ? \; \frac{\log L_n \; ? \; \log \; ?}{\log L_n \; ? \; \log L_{n?1}} \qquad (1)$$

where, PI is the plastochron index of the plant and *n* is the node number on which the compound leaf is attached to the plant.



SIMPLE LEAVES
PLANT A

COMPOUND LEAVES
PLANT B

$$PI = n + \frac{\log L_n - \log \ell}{\log L_n - \log L_{n-1}} = n + \frac{\log \left(\frac{a+b}{2}\right) - \log \ell}{\log \left(\frac{a+b}{2}\right) - \log \left(\frac{c+d}{2}\right)}$$

Fig. 7.1: Measurements used for calculating PI in plants with simple leaves (A) or compound leaves (B).
Equivalent determinants used in each case, are shown on the diagrams for Ln, Ln+1, n+1 and n respectively from both plants are indicated in the formula.

> The arbitrarily determined reference length, ?, relates to other parameters such that, $L_{n-1}, \left(\frac{c+d}{2}\right) \geq \ell > L_n, \left(\frac{a+b}{2}\right)$ and, all other lengths of leaves or leaflets below *n*, are longer or equal to the reference length, ?.

The PI formula which was developed and used in this thesis allowed exploration of the quantitative and qualitative aspects of growth and development. Of great importance was that all data and observations could be and were related directly to the plastochron index. These included the effects of different CO_2 and nitrogen growth conditions on PI. Quantitative analyses were focussed on growth rate, whilst

qualitative analyses centred on the investigation of leaf ontogeny, based on sink-to-source transition of leaflets and, associated with this, anatomical studies at the light and electron microscope level, all on leaflets of the same leaf plastochron index (LPI) in all experiments.

7.2 Assimilate movement – sink to source transition

As leaves unfold and start to expand, the transport of assimilates changes from being sink orientated to bi-directional (transition) and, at full maturity, to source orientated. The sink/source state of leaflets was analyzed in Chapter 4 based on the transport of the symplasmically-transported fluorescent probe, 5,6-carboxyfluorescein, between attached leaflets on the same plant. The results demonstrate that young leaves remain strong sinks up until LPI 0, after which sink-source transition occurs in a basipetal direction in the lamina. This process continues up to LPI 1.8 hereafter leaflets become strong source systems by the LPI 2 stage. These results offer convincing support for the actual age or state of the leaf, as sink, transition and source development progresses.

Of major interest was the observation of a well-developed cross-connected phloem supply between paired leaflets in peas, and the subsequent connection of this supply to the petiole and the stem vascular supply. The contention is that this cross-connection appears to be involved in, and utilized for transporting assimilates between

paired leaflets, irrespective of their sink/source state. These results are convincing evidence that transport across the vascular connections between leaflets is autonomous, and independent of the sink/source state of the leaflets. Furthermore they suggest that these connections could be independent of the source to sink gradient. Thus, the data which I have presented here, strongly support the presence of a modular transport system, first reported by Kaitaniemi and Honkanen (1996) and subsequently by Yang and Midmore (2004). The modular transport system apparently ensures that re-allocation and balancing of assimilates takes place first between paired leaflets, ensuring load-balancing of the local transport system before any export to other younger (sink) regions can occur.

The sink/source states of the first pair of leaflets in relation to LPI are shown in Table 7.1. This table is useful as it allowed prediction of the sink/source state of the first pair of leaflets on the compound leaves at any node of a pea plant of a known plastochron index.

Table 7.1: Shows the plastochron index and its relationship to sink/source state in peas.

LEAF PLASTOCHRON INDEX (LPI)	SINK/SOURCE STATE
0 – 0.4	**Sink**
0.5 – 0.8	**Transition (tip region of lamina)**
1 – 1.4	**Transition (mid region of lamina)**
1.6 – 1.8	**Transition (basal region of lamina)**
2 AND ABOVE	**Source**

Using the diagram in Fig. 7.2 in which two plants with different PI values demonstrate the usefulness of the data in Table 1. The diagram in Fig. 7.2 of two plants demonstrates the real conditions of a plant's current status. For example, plant A at PI 6.0 (Fig. 7.2A) and plant B at PI 6.5 (Fig. 7.2B): both plants have leaflets at corresponding LPI values (as shown on the diagram) as determined from the LPI formula on page 141. Figure 7.2A shows that leaflets at LPI -1 and 0 are sinks while leaflets at LPI 2 and 3.0 have transitioned fully and are now sources. Only the leaflets at LPI 1 are in sink-source transition state. In contrast, the leaflet which is at LPI -0.5 is in a sink state and leaflets at LPI 2.5 and 3.5 are in the source state. Leaflets between LPI 0.5 and 1.5 are in transition, there are 2 leaves in which the first pair of leaflets are under transition in the plant in Figure 7.2B. in other words 1-2

leaves will be under transition in a pea plant at a given time. From the literature, it is apparent that some variability exists in the number of consecutive leaves which have been reported to be in sink-to-source transition at any one time. For example, four successive leaves were found to be simultaneously in transition in *Moricandia arvensis* L. (Gagnon and Beebe 1996a), sugar beet and tobacco (Turgeon and Webb 1973) while squash was reported to have only one leaf in sink-to-source transition at any given time (Turgeon and Webb 1973). The duration of sink-to-source transition appear also to be species-dependent. For example, Gagnon and Beebe (1996a) have shown that sink-to-source transition took approximately 2.5 plastochrons in *Moricandia arvensis* L., while results of the experiments reported in this study showed that sink-to-source transition in *P. sativum* takes about 2 plastochrons. Clearly though, growth conditions could influence the rate of growth of the species, and hence, the number and development rate at which leaves are produced.

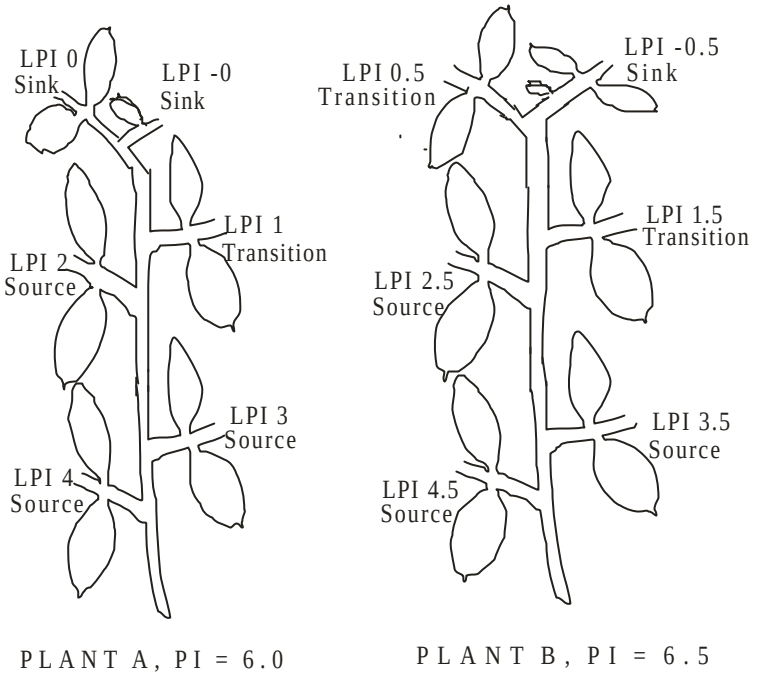


Fig 7.2: The sink/source state of leaflets at each node on Plant at PI 6.0 (A) and Plant at PI 6.5(B).
Note that LPI values of attached leaflets are determined from the LPI formula, LPI=PI-n, where n is the node number of the leaf bearing the leaflets. Leaves on node 1 and 2 on pea plants are minute and have therefore been left out.

7.3 Anatomical studies

The anatomical study of leaf ontogeny in relation to plastochron index (Chapter 5) showed that structural development in peas is directly related to LPI. Various authors have also related leaf development in *Xanthium* (Maksymowych 1959; Maksymowych and Erickson 1960); shoot apex development in *P. sativum* (Lyndon 1968); petiole development and xylem differentiation in *Xanthium* (Maksymowych and Maksymowych 1982) to plastochron index. Light microscopy studies of the cross section of the leaf lamina illustrated the changes in the different layers of the lamina during leaf expansion. Results suggested that once formed, the lamina only becomes larger due to the expansion of the cells and the reaction of the epidermis and counter reaction of the mesophyll cause the formation of intercellular spaces. Therefore the young leaf with compact mesophyll organization at LPI 0 contains numerous intercellular spaces between the mesophyll cells by LPI 1. Tichá (1997) showed that the compact mesophyll organization inherent in young leaves limits CO_2 transfer and invariably the rate of photosynthesis. The light microscopy studies support the fluorescence study, which demonstrated that leaflets at LPI 0 are strong sinks, importing assimilates from other leaflets due to the inadequate production of assimilates required by the leaflets for growth and development at LPI 0.

In Chapter 5, I have suggested as have others, that plasmodesmata and phloem sieve tube development in large minor veins were not directly related to LPI. Apparently functional plasmodesmata and mature phloem sieve tubes were found in class IV veins in the upper region of young and older leaflets. The number of mature phloem sieve tubes per vein increased with LPI. Most class V veins were still undergoing division at LPI 0 and their phloem sieve tubes did not show signs of maturity until LPI 1. The development of the transfer cells appears to be related to LPI and specifically the development of wall ingrowths in the mature transfer cells. My data suggest that the density of wall ingrowths produced increases as transfer cells age from LPI 0 to LPI 1. This seems to be related to the increase in the production of assimilates for export as other workers (Gunning *et al*. 1968; Peterson and Yeung 1975) have shown that development of wall ingrowths in peas is synonymous with the commencement of export. Again, my study already supports this.

It would be expected that export would have commenced in large minor veins by LPI 0 since anatomical studies showed that they contain already- mature phloem sieve tubes and transfer cells to qualify the veins as being classified as mature (Turgeon and Webb 1976). If this is so, then the fluorescence data (Chapter 4) that the tip region of the lamina used in the anatomical analyses is still importing at LPI 0 may not be correct. However, both results find support in the work of

Turgeon and Webb (1976) which suggest that the mature larger minor veins are not utilized for export before the smaller minor veins mature, as export is limited by another step in the vein-loading transport system. Fellows and Geiger (1974) suggest that the attaining of a threshold concentration of solutes in the phloem of the minor veins is that essential step. Therefore, the anatomical analyses and fluorescence studies are confirmatory and provide further evidence that lamina regions become mature prior to the transition from sink to source as suggested earlier by Fellow and Geiger (1974).

7.4 PI and growth rate

The quantitative analyses carried out using the plastochron index was based on the fact that measurements taken to determine PI as a time scale can also be carried out over time on a single plant and that the data accumulated can then be used to determine leaf appearance rate and leaf elongation rate. The effects of growth conditions (CO_2, nitrogen and nodulation) on these growth rates in *P. sativum* were analyzed and the results, which were presented in Chapter 6, showed the difference between plants grown under different growth conditions. Results showed that short-term exposure to elevated CO_2 induced plant growth, irrespective of treatment. Long-term elevated CO_2 treatment did not affect rate of leaf appearance (RLA) in nodulated plants irrespective of mineral N supply but induced RLA in non-nodulating plants supplied with mineral N (Fig. 6.4). Supplied N induced leaflet

elongation rate significantly under both ambient and elevated CO_2 in nodulated and non-nodulated plants (Fig. 6.5). Leaflet elongation rate was not significantly affected by nodulation but was increased by high CO_2 (Fig. 6.5). The results reported here, show that a CO_2 level of as high as 1000 µmol mol^{-1} may not significantly affect the growth of nodulating *P. sativum*. The data suggest that elevated CO_2 will enhance canopy size, provided adequate soil N is available. In addition, canopy size will be significantly increased under elevated CO_2 in non-nodulating plants, provided adequate soil N is applied. Clearly, the data presented in this chapter provides significant evidence of differences induced by chronology, coupled with significant differences induced by N supply in non-nodulating and nodulating *P. sativum*. Using the plastochron index is strongly supported by the data for all physiologically or ecophysiologically-orientated experiments using variables that affect plant growth.

7.4 Aligning qualitative and quantitative analyses based on plastochron index

There was no apparent noteworthy difference in the sink/source state or anatomy of plants from different CO_2, nitrogen and nodulation treatment conditions except that plants grown under high [CO_2] accumulated starch at an earlier age. The fact that leaflets at the same LPI under different growth conditions are not very different, clearly demonstrates that plants of the same PI value are at the same

developmental state, and that this appears to be independent and irrespective of the growth conditions imposed. However, the results of the quantitative analyses showed that plant growth rates were affected by [CO_2], nitrogen and nodulation treatment conditions. The difference between qualitative and quantitative results therefore brings to mind my probe (highlighted in the general introduction) into the possibility of reports on the differences in growth and development of plants under different growth conditions, rather being a function of differences in plastochron index of plants; and therefore the question:

'Are plants/leaves of the same chronological age under different growth conditions, at the same PI value and vice versa?'

can be answered by comparing the data in Figs. 6.1A, 6.2A and 6.3A. These figures show that plants at same chronological ages do not have the same PI values under different growth conditions. As plastochron index is a developmental scale, it is obvious that plants of same chronological age must be at different developmental states under different growth conditions. This inference can be further illustrated using LPI values of leaves of the plants under different growth conditions. Table 7.2 shows the leaf plastochron indices of leaves on node 6 at the same chronological age under different growth conditions as extrapolated from the data presented in Figs. 6.1A, 6.2A and 6.3A. If the results of the qualitative analyses are compared to those of the quantitative analysis, it is obvious that leaflets of same chronological age under different growth conditions are physiologically and

morphologically different. For example, fluorescence studies (Chapter 4) showed that leaflets attain full source states by LPI 2; if this is projected to the nearest value in Table 7.2 (bold values), it becomes evident that leaflets on similar node on plants of the same chronological age under different growth conditions do not in most cases attain full source states at the same time (for example. see bold values of REn and RAn). Plants/leaves of the same chronological age are qualitatively different because they are simply not at the same plastochron age. Therefore, most changes in plant structure and function observed under different growth conditions can be related simply to changes in plastochron index.

Table 7.2: Matrix comparison of the leaf plastochron index (LPI) of leaves of *P. sativum* attached to node 6 under all treatments: nodulating plants supplied with N- nutrient solution under elevated (REn) and ambient (RAn) CO_2.
Nodulating plants supplied with N+ nutrient solution under elevated (REN) and ambient (RAN) CO2; and non-nodulating plants supplied with N+ nutrient solution under elevated (rEN) and ambient (rAN) CO2. Leaflets at the bold LPI values are in the source state.

Nodulating					Non-nodulating	
No mineral N supply		With mineral N supply				
Days after plant germination	Elevated (REn)	Ambient (RAn)	Elevated (REN)	Ambient (RAN)	Elevated (rEN)	Ambient (rAN)
11	-0.02	-0.75	-0.04	-0.36	-0.01	-0.74
12	0.42	-0.52	0.53	0.20	0.53	-0.39
13	0.72	-0.20	1.22	0.80	1.16	0.09
14	1.11	0.10	1.73	1.30	1.68	0.63
15	1.42	0.39	2.19	1.77	2.16	1.25
16	1.77	0.83	**2.56**	2.17	**2.70**	1.67
17	2.23	1.23	**2.92**	**2.50**	**3.17**	2.08
18	**2.65**	1.68	**3.22**	**2.84**	**3.62**	2.49
19	**3.03**	2.06	**3.63**	**3.22**	**4.01**	**2.83**
20	**3.41**	2.45	**3.96**	**3.56**	**4.40**	**3.16**

The variation in the plastochron index of plants or leaves of same chronological age under different growth conditions highlights and underscores the statement by Erickson and Michelini (1957) that plant organs which are at the same chronological age are not at the same physiological state under different growth conditions. Reports showing differences in physiological activities of plants grown under different growth conditions are therefore hardly surprising, as analyses are usually carried out with the comparison of the activities of organs such as leaves of same chronological age. Therefore, differences in plant structure or function under different growth conditions may be due to changes in the plastochron value of the plant under such conditions.

7.5 Relevance of research to the future

The greatest usefulness of the PI concept according to Larson and Isebrands (1971) is in the study of biochemical and physiological processes where it is absolutely necessary to maintain uniformity among plants from many different harvests and to relate these processes to morphological events. PI can thus minimize population variances by using the determined PI values to adjust for differences in morphological development. Since it is non-destructive, PI will aid in the selection of the proper stem position for sampling within a plant (Larson and Isebrands 1971). The prediction of biological events using LPI is a powerful tool as it also permits one to determine how

shifts in plant development, for example, due to different treatments or environments, affect plant structure. Simply put, growth analysis based on PI can be used to predict differences in structure or function of plants under different growth conditions.

For example, all that needs to be done in future is to calculate the PI of a pea plant under any growth condition. Using the data in this thesis and that which has been published, the relative sink/source state of all the leaves on the plant can therefore be predicted. As sink-to-source transition takes about 2 plastochrons, the number of days in which leaflets are in transition from sink to source would be calculated by multiplying plastochron duration of the plant in days by 2. Though it is acknowledged that the prediction of quantitative data is subject to stringent limitations, Larson and Isebrands (1971) and others have argued that the prediction of qualitative biological events according to LPI can be both (highly) accurate and extremely useful. This was found to be the case in this thesis as well.

7.6 Future studies

The characteristic predictable movement that occurred between leaflet pairs, irrespective of the sink-source state of the leaflets and the inferred hypothesis of modular supply, demand more investigation and interpretation. Much of what has still to be done requires sophisticated microscopy techniques to examine in situ events. It is possible that the

results might be enhanced by the use of confocal microscopy for example, to resolve the detail image of the leaflets and vascular supply before load-balancing hypothesis can be examined further. The emergence of lateral branches in *P. sativum* plants under elevated CO_2 conditions is also an issue requiring further investigation; but again my observations are beyond the scope of the thesis and would require access to facilities in well-established plant growth regulation laboratories. The lateral branches which only emerge under high [CO_2] are possibly acting as strong redirected sinks. The effect of the lateral branches emergent under high [CO_2] could be investigated fully using $^{14}CO_2$-based experiments coupled with Confocal microscopy.

Finally if I may quote the statement by Prof. Reto J Strasser (Laboratory of Bioenergetics, University of Geneva, Switzerland) after he reviewed one of the papers published from this research: "Physiology is an important part of Botany and in vivo diagnostics is in the centre of interest in Life Sciences. The paper about the Plastochron Index and the extension of its application to leaflets is modern with a value we should not underestimate. The paper is not the invention of this generation, but it is a well done work, which refreshes old excellent ideas of plant physiological quantification. It is a paper for all the scientists who are interested in non destructive growth of legumes especially peas. Forthcoming papers on PI and peas should cite this equation (maybe by adding: based on the equation for leaves

by …..). This is the new and smart part of the MS and I'm happy to see that soon in the SAJB". I have taken an old concept – leaf plastochron index and have shown using various quantitative and qualitative measures that LPI is highly relevant to all experiments and growth observations, providing a simple, yet powerful tool in the assessment of the actual age and stage of development of the humble pea plant.

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