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A re-evaluation of plastochron index determination in peas — a case for using leaflet length

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The plastochron index (PI) is a measure of plant growth and can be used to determine growth rate, based upon appearance of successive leaves on the axis of the plant. PI should under ideal growth conditions be a regular event and should be predictable with a relatively small error of a few hours. PI has been variously calculated in peas, and each method reported has had with it a number of problems that do not allow for reasonable prediction of PI. Internode length varies greatly and is dependent upon the variety, which may be short- or long-stemmed; thus this parameter is not ideal for determining growth rate or plant age. This paper reports our findings on PI using the average length of the first pair of leaflets on each node. Early leaflet growth in peas occurs exponentially and the early stages of growth of successive pairs of leaflets occur at the same relative growth rate. Given that growth of leaflets during early development can be measured successfully, we propose the use of leaflet growth as a measure of the plastochron index in peas. Our results suggest that plant age is best expressed using the plastochron index, which is a measure of the time interval between the initiations of successive events — in the case of peas, of successive pairs of leaflets.

Abbreviations: DAG = days after germination, PI = plant plastochron index

Introduction

The study of plant growth and development is usually carried out using histological examination, chemical analysis, metabolic or molecular studies and must be related to time if they are to have meaning in developmental terms. Growth-related data are usually plotted against the plant's chronological age. However, it is clear that 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. Variability can only be reduced when plants are not only genetically uniform but also under same conditions of growth (Erickson and Michelini 1957).

Erickson and Michelini (1957) developed a numerical index called the plastochron index, for measuring the developmental status for the plant of interest in which growth observations were related to time directly using an index. According to Erickson and Michelini (1957), a plastochron is broadly defined as the interval between corresponding stages of development of an organ in succession, where the organ in most cases is the leaf. A plastochron can serve as the unit of developmental scale, when successive plastochrons are equal in duration (Erickson and Michelini 1957). These authors went on to develop the formula for plastochron index (PI) using *Xanthium*, defined as:

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

where n is the serial number (counting from the shoot base) of that leaf which just exceeds a predetermined reference length (λ) in mm; log L_n is the natural logarithm of the length of the leaf n; and log L_{n+1} is the natural logarithm of the succeeding leaf with a length that is less than λ.

Erickson and Michelini (1957) showed that PI is linear over time. The inverse of the slope of the linear graph gives the average duration of the plastochron. A plant is n plastochron old when the length of the leaf n is exactly λmm. It is noteworthy that λ is the same reference as R used by other authors such as Van Heerden *et al.* (2004).

PI thus provides a morphological time scale, which has proved to be more reliable than chronological age in studies relating morphological and physiological development of a whole plant, or plant organ (Lamoreaux *et al.* 1978, Van Heerden *et al*. 2004). However, because the pea leaf is awkward to measure, as each leaf axis is terminated by tendril(s) and the developing leaf in any case is tightly enclosed by the stipules during the early stages of leaf expansion, Erickson and Michelini (1957) proposed the use of internode length in the calculation of the PI in peas rather

than leaf length. Leaf length is replaced by internode lengths in equation (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.

Studies involving the use of plastochron age in *Pisum 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, in a not too clear manner. Lyndon (1968) defined the *n*th leaf primodium produced as being *n* plastochron old, and each of nine morphologically recognisable stages of primodium development as 0.1 plastochron unit. 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 centre 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. All of these methods are either complicated or involve damaging the plant. However, 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 (Erickson 1976).

Preliminary experiments showed 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 favourable for all varieties. The aim of the research reported here was to explore an alternative parameter for determining PI in *Pisum sativum* L., which would allow replicable results and which would remain within the constraints originally proposed by Erickson and Michelini (1957).

Materials and Methods

Germination and growth of plants

Seeds of *Pisum sativum* var. Greenfeast were sown in potting soil (Greenfingers, South Africa) in pots (185mm x 185mm, 165mm deep). Four seedlings were transplanted per pot upon germination. Five grams of slow-releasing fertilizer (NPK 2:3:2; Wonder Horticultural Products, Johannesburg, South Africa) was added to the soil in pots prior to transplanting seedlings. Pots were irrigated with full strength Long Ashton nutrient solution (Hewitt 1966). Plants were germinated and grown in a growth chamber (Conviron Model S10H, Controlled Environments Ltd, Winnipeg, Canada) under 25/18°C day/night at 16h photoperiod with CO_2 maintained at 360µmol mol^{-1} with insignificant fluctuations within ±15µmol mol^{-1}. CO_2 was monitored using the 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 400–700nm) was set, such that it was about 250µmol m^{-2} s^{-1} when measured 20cm 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.

Measurements

The first true leaf of the pea plant (with oval shaped leaflets and tendrils) is borne on node 3. Node numbering was taken from the base of the plant with the cotyledon attached to node zero, while nodes 1 and 2 bear scalar leaflets. The first true leaves on nodes 3 and 4 are nearly opposite and remain of approximately equal length throughout their development. According to Erickson and Michelini (1957), this is common in many dicotyledonous seedlings. It is therefore inappropriate to calculate the plastochron index before the plant has entered the second plastochron; that is, when leaves have been produced on node 5 (Erickson and Michelini 1957). In order to avoid much error, whole leaf, leaflet and stipule length measurements were therefore recorded from the first pair of leaflets attached to node 5. The internodes of the plant were numbered from the base up, each internode taking its number from the leaf that subtends it. Since the internode subtended by node 5 as at when measurement commenced had not been succeeded by another internode (on node 6), internode length measurements were taken from the internode subtended by the second true leaf (node 4). Measurements were made using an electronic digital caliper at the same time every day, throughout the vegetative stage of growth.

Statistical analysis

Measurements were recorded for each node. Data were analysed for each plant. Descriptive and regression analysis was carried out using Microsoft Excel 2000. One-way analysis of variance (ANOVA) was carried out at the 5% level of significance using 10 replicates and experiments were repeated twice.

Results and Discussion

The determination of the plastochron index (PI) using internode length and stipule length as well as the more general conventional use of leaf length outlined in the original proposal by Erickson and Michelini (1957) for the calculation of PI is reported briefly below.

Internode length

The internode lengths of plants 15 days after germination (DAG) are shown in Table 1. Table 1 shows that there is considerable variation in number of nodes produced as well as in node length, by 15 DAG in Greenfeast pea plants grown under controlled environmental conditions. If the reference length (20mm) suggested by Erickson and Michelini (1957) is applied, then only plants 3 and 7 (data in bold typeface, Table 1) could be used to calculate plastochron age, and it is unrealistic (and misleading) to

Table 1: Internode length (mm) of 10 *Pisum sativum* var. Greenfeast plants grown under controlled environments 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 number					
	4	5	6	7	8	9
Plant 1	16.12	18.35	12.02			
Plant 2	18.36	18.01	13.39	13.02	15.12	15.13
Plant **3**	**22.35**	**18.24**	**17.00**	**17.19**	**8.08**	
Plant 4	16.78	16.58	15.18	19.07	14.24	
Plant 5	18.54	16.26	15.92	16.13	14.56	
Plant 6	18.13	16.94	16.01	15.03	12.14	
Plant **7**	**21.02**	**17.27**	**17.03**	**17.59**	**15.86**	
Plant 8	18.68	13.46	13.32	15.04	12.46	
Plant 9	18.92	16.00	13.29	13.13	10.62	
Plant 10	17.08	14.10	15.01	14.17	14.14	4.07
Mean	18.60	16.52	14.82	15.60	13.02	9.60
Variance	3.56	2.76	2.99	4.28	6.11	61.16

state that these plants were 3.48 and 3.23 plastochrons old by 15 DAG. Furthermore, neither will reach the fourth plastochron for an indeterminate time period.

A second option could be to use a different reference length. However, two criteria need to be met before choosing the reference length:

1. The reference length must be such that the length of internode *n* is equal or greater than the reference length, while length of internode *n*+1 is less than the reference length.
2. All internodes developed before internode *n* must be longer than the reference length.

Fulfilling both criteria in all plants sampled proved to be impossible, as illustrated in Table 1. No matter what reference length is 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 shorter than the chosen reference length. Criterion 2 cannot therefore be met using this approach.

Stipule length

Using stipule length measurement proved to be equally difficult. Stipules sometime fold inwards or outwards, requiring manual unfolding that resulted in injury and reduction in the rate of elongation of the stipule after manipulation. The serrate base of the stipules also clings very close to the other pair and the node (base of petiole), making it sometimes difficult to determine the position of the lower end of the stipule without causing injury.

Leaf length

The pea leaf is composed of a pair of stipules, pair(s) of leaflets and terminates with one or more tendril(s). The length of the whole leaf is therefore difficult to measure, due to tendril coiling. Uncoiling the tendril during measurement must therefore lead to tendril damage, which would effectively retard or prevent future elongation of the leaf being measured. Perhaps more important is that tendril growth is not time- but proximity-based, and it is therefore highly dependent on the proximity of the tendril to a supporting object or structure. In other words, its length depends on how close the nearest support structure is and elongation growth ceases 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 time.

Leaflet length

Pea leaflets are analogous to leaves in most dicotyledon species and it therefore seemed logical to use leaflet length to determine plastochron age. Measuring leaflet length proved to be easier to accomplish as leaflets can be manipulated into a position 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', we explored the plastochron in *Pisum sativum* as the interval between initiations of successive first pairs of leaflets.

We determined the appropriate reference length for estimating PI. Leaflet length measurements were taken for a week on a daily basis from 11 DAG. Mean leaflet length against time was plotted for each plant (Figure 1). Figure 1 shows the progressive sequence of leaflet development. Leaflets show a typical growth and enlargement pattern, before transitioning the log to a lag growth phase. The r^2 values (indicated in the legend) indicate an almost perfect linearity during log phase and the slope of the elongation rate of leaflets (in 10 replicate plants) on nodes 6–10 of the 10 plants sampled averaged 3.26 ± 0.29 with no significant difference at P = 0.05. This shows that early leaflet growth occurs at the same relative rate. A leaflet on leaf *n* is about 20mm long by the time the following leaf *n*+1 unfolds (highlighted on Figure 1 by vertical dashed lines); therefore, a reference length of 20mm was realistic. The adapted formula for PI for *P. sativum* would therefore be:

$$PI = n + \frac{\log L_n - \log 20}{\log L_n - \log L_{n+1}} \qquad \text{Equation (2)}$$

where L_n and L_{n+1} (expressed in mm) and the reference length 20mm refers to *leaflet-length* instead of the length of the *leaf* as indicated in Equation (1).

PI was calculated for each plant per unit time from 11 DAG, and PI vs time was plotted for each individual plant. Figure 2 shows two examples in which variation in the rate of change of PI vs DAG was noted with resultant changes in regression values. Figure 2 further shows that the relationship between the PI and time is linear for plant 1, but that this is not completely so for plant 2. These data find support in Erickson and Michelini's (1957) results, as these authors reported similar variability using *Xanthium*. After about PI 11, a decrease in the slope of the curve was noted in some pea plants. Erickson and Michelini (1957) observed a similar decrease in the slope of the curve for *Xanthium* by about PI 13, and suggested root binding of the plant as the cause. The plants used in this study were also cultivated in pots, which could limit root growth. A straight line was fitted

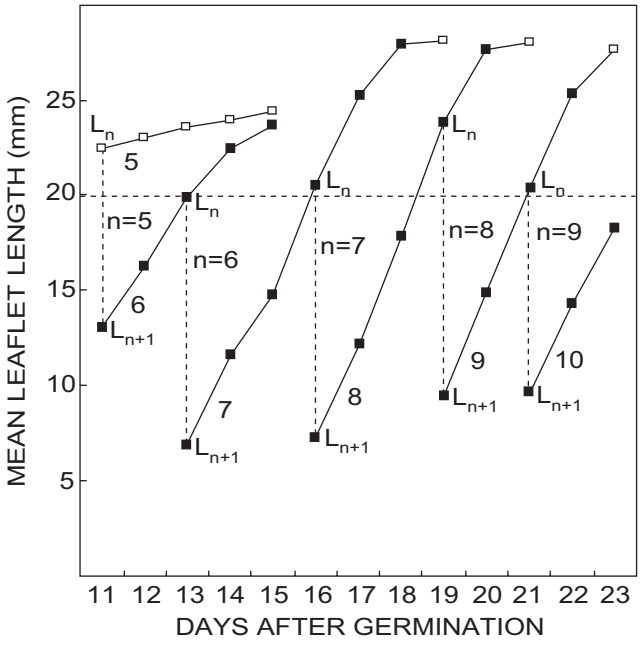


Figure 1: Mean lengths of successive pairs of leaflets of a *Pisum sativum* L. plant plotted against time. Each growth curve applies to leaflets on a particular node (node number indicated beside curve). Equations for the period of linear leaflets' growth (elongation in mm per day; points marked with full symbols) for each node are: $y_6 = 3.162x + 10.02$, $r^2 = 0.9964$; $y_7 = 4.348x - 6.1073$, $r^2 = 0.9923$; $y_8 = 5.261x - 24.352$, $r^2 = 0.996$; $y_9 = 4.686x + 0.5483$, $r^2 = 0.9816$; $y_{10} = 4.31x - 37.657$, $r^2 = 0.9986$. Horizontal dash line indicates length in which leaf *n*+1 becomes measurable and vertical dash lines indicate values needed to calculate plastochron index per time

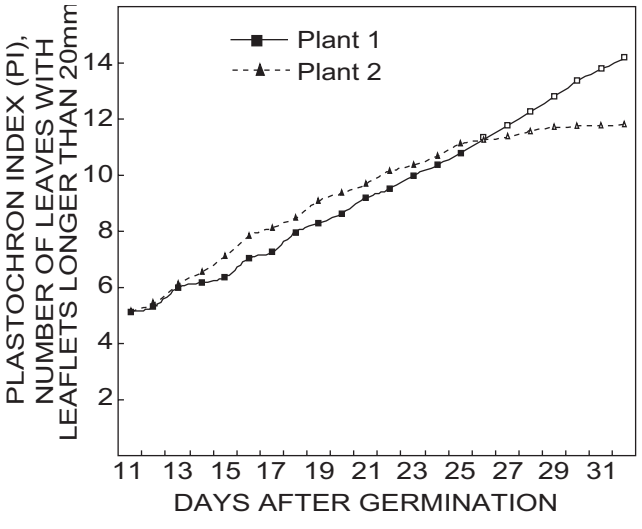


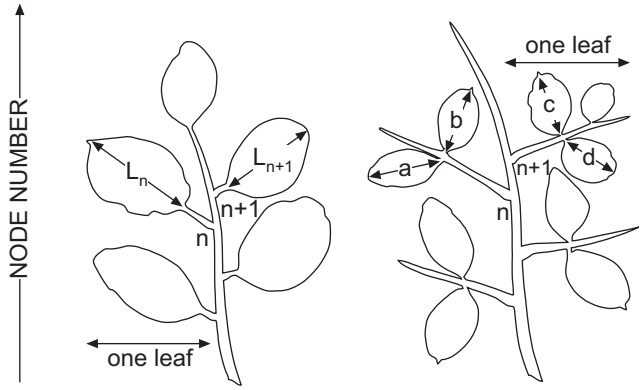
Figure 2: The plastochron index of two *Pisum sativum* var. Greenfeast plants. The equations for the linear regression for the period of DAG indicated with full symbols (11–20 DAG, about PI 11) are: $y_1 = 0.4157x + 4.5575$, $r^2 = 0.9959$; $y_2 = 0.432x + 4.904$, $r^2 = 0.9881$

by least squares to these data up to about PI 11 in all plants, as was the case in the data reported by Erickson and Michelini (1957).

The SE of regression lines for the estimated PI for both plants (Figure 2) was calculated to be approximately 0.01 while the slopes of the fitted lines were 0.42 day^{-1} and 0.43 day^{-1} respectively. The average duration of the plastochron is therefore 2.38 and 2.33 days respectively. The standard errors for both plants were about 0.01 plastochron, which is approximately 0.02 days. Extrapolating further, the standard error of PI is less than one hour. For the other plants on which this paper is based (data is not shown), SE was less than 2h overall. Our data therefore compare favourably with Erickson and Michelini's (1957) data where an SE of up to 7.63h was recorded for some *Xanthium* plants sampled. Calculating PI using leaflet length is, in our opinion, thus justified.

Leaflet length was used to determine PI using the Black-eyed Susan pea variety. This variety has very long stems and an internode that reaches about 30mm before the subsequent internode become visible. Succeeding internodes are enclosed in the stipule along with the new leaf for a period. Using the method described for Greenfeast, the average plastochron duration of the Black-eyed Susan plants was 3.96 ± 0.31 days with errors of less than 3h.

Where plants have compound leaves such as the pea plants used here, we suggest using combined averaged data for both pairs of leaflets in the equation, as illustrated in



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

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

Figure 3: Measurements used for calculating PI in plants with basic leaves (A) or leaflets (B). Equivalent values from both plants are indicated in the formula

Figure 3 below. The difference in the approach needed for the use of leaflet length in plants like peas as against the conventional use of leaf length in most plants is illustrated in Figure 3. In plant A, the length of a leaf *n* that is longer or equal to λ and that of its succeeding leaf *n*+1 whose length is less than λ are used in calculating PI. On the other hand, PI is calculated in the pea plant B using the *average* length of leaflets a and b on leaf *n*, which is longer or equal to λ, and that of leaflets c and d on the succeeding leaf *n*+1, which is less than λ. It should be noted that only the first pairs of leaflets are measured (see leaf *n*+1 of plant B, Figure 3).

Mean length of successive pairs of leaflets in determining plastochron age of *Pisum sativum* satisfies all the criteria in which leaf length is used for the determination of plastochron age, as stated in Erickson and Michelini (1957) and subsequently by Lamoreaux *et al.* (1978). These criteria are that:

1. Early leaflet growth occurs at an exponential rate — Figure 1 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.
2. Early growth of successive leaves on a single plant occurs at the same relative rate — the graph for the successive pairs of leaflets (Figure 1) has similar slopes and occurs at approximately the same periodicity.
3. 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 *Pisum sativum* L. differs only in a few hours, which is less than that achieved by Erickson and Michelini (1957). Erickson and Michelini (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.

Based on the data presented here, the plastochron in *Pisum sativum* is thus defined as the time between initiations of *successive first pairs* of leaflets. The use of the mean length of successive leaflets in determining PI is appropriate to other plants with compound leaves and is advocated, provided the same leaflet pair (we suggest basal pair) is used throughout.

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