Biometrics 66, 159–168
March 2010
DOI: 10.1111/j.1541-0420.2009.01237.x

Nondestructive, Stereological Estimation of Canopy Surface Area

Dvoralai Wulfsohn,[1,*] Marco Sciortino,[2] Jesper M. Aaslyng,[3] and Marta García-Fiñana[4]

[1]Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen, DK-2630 Taastrup, Denmark

[2]Dipartimento di Scienze e Tecnologie Agromabientali, Università Degli Studi di Bologna—Alma Mater Studorium, 40127 Bologna, Italy

[3]AgroTech. Institute for Agro Technology and Food Innovation, DK-2630 Taastrup, Denmark

[4]Centre for Medical Statistics and Health Evaluation, University of Liverpool, Liverpool L69 3GS, United Kingdom

*email: dw@life.ku.dk

Summary. We describe a stereological procedure to estimate the total leaf surface area of a plant canopy in vivo, and address the problem of how to predict the variance of the corresponding estimator. The procedure involves three nested systematic uniform random sampling stages: (i) selection of plants from a canopy using the *smooth fractionator*, (ii) sampling of leaves from the selected plants using the *fractionator*, and (iii) area estimation of the sampled leaves using *point counting*. We apply this procedure to estimate the total area of a chrysanthemum (*Chrysanthemum morifolium L.*) canopy and evaluate both the time required and the precision of the estimator. Furthermore, we compare the precision of point counting for three different grid intensities with that of several standard leaf area measurement techniques. Results showed that the precision of the plant leaf area estimator based on point counting is high. Using a grid intensity of 1.76 cm^2/point we estimated plant and canopy surface areas with accuracies similar to or better than those obtained using image analysis and a commercial leaf area meter. For canopy surface areas of approximately 1 m^2 (10 plants), the fractionator leaf approach with sampling fraction equal to 1/9 followed by point counting using a 4.3 cm^2/point grid produced a coefficient of error of less than 7%. The *smooth fractionator* can be used to ensure that the additional contribution to the estimator variance due to between-plant variability is small.

Key words: *Chrysanthemum morifolium* L; Coefficient of error; Fractionator; Nested cluster sampling; Point counting; Smooth fractionator; Stereology; Surface area; Systematic sampling; Variance prediction.

1. Introduction

Leaf area is an important parameter of canopy structure. Its determination is essential to establish the size of the assimilatory system, plant growth rate, and the assimilation rate of a leaf or canopy. Leaf area index (LAI), defined as the ratio of the total area of leaves on plants to the area of ground covered by the plants (Watson, 1947), influences the amount of photosynthetic photon flux (photons with wavelengths between 400–700 nm) intercepted by the canopy. Plant height and canopy structure have little influence on light penetration for very low LAIs, whereas they greatly influence light penetration for LAI values above 1.5 (Heinicke, 1966).

Nondestructive measurements are highly desirable because it is then possible to repeat measurements on the same plants through the growing season providing information related to physiological processes and their rates. In practice it is important to balance the cost and time required compared to the estimation accuracy. Leaves may have complex shapes, making leaf area determination more difficult, time consuming, and subject to larger errors. Area estimation of flat and wide leaves is easier compared to small or narrow ones, such as conifer needles or grass leaves (Long and Hällgren, 1993). One approach has been to develop empirical relations between leaf area and linear leaf dimensions (e.g., Robbins and Pharr, 1987; Ray and Singh, 1989; Jensen, Rosenqvist, and Aaslyng, 2006). A number of high resolution commercial leaf area meters that optically enlarge the object for measurement are available on the market for either laboratory use (usually destructive on the plant) or field measurements. Instruments that indirectly assess canopy leaf area by measuring light transmission through canopies or shadows cast by canopies (Welles, 1990; Wünsche, Lakso, and Robinson, 1995; Sellin, 2000; Jonckheere et al., 2004) are also widely used. These indirect methods all require calibration for a set of plants of interest. Attempts have also been made to estimate leaf area by taking images of entire plants from two mutually perpendicular directions (Baker, Olszyk, and Tingey, 1996; Bignami and Rossini, 1996). The attractiveness of image analysis and sensors for indirect measurements are that they are nondestructive, precise, and rapid; however, imaging of the projections of entire plants can produce substantial and unknown biases because of occlusions, parallax effects, and nonuniform leaf orientations.

Another approach is to draw upon principles of stereology to derive nondestructive procedures to measure parameters related to the size and number of complex vegetal structures. The classic work of Warren Wilson and Reeve (1959) and Warren Wilson (1960) to estimate LAI and related parameters using linear probes are examples of a stereological principle. A number of recent studies have reexamined the use of methods based upon point counting (among others) for estimating foliage areas and densities in forestry (Radtke and

Bolstad, 2001; Bréda, 2003; Jonckheere et al., 2004). Our review of the botanical literature indicates that estimates from sampling techniques are generally reported without determining the estimator variance. Estimates are commonly claimed to be accurate on the basis of comparisons with measurements taken using other instruments that have unknown biases.

The goals of this article were to develop a stereological procedure for nondestructive estimation of the total leaf area of plant canopies and to evaluate its performance for chrysanthemum. The proposed method is suitable for sampling of plants having a suitably large leaf internodal length so that branching elements can be distinguished, and for measurement of surface area of leaves that can lie in a two-dimensional plane.

2. Stereological Design

2.1 *Introduction to Stereology*

Stereological methods provide a stochastic approach to measurement (Baddeley and Jensen, 2004). The material or object is regarded as the population of interest, and a section or projection of the object is a sample from which statistical inferences can be made about the geometric structure of the object. Stereological methods have found wide application in the biomedical, materials, and geological sciences for estimating microstructure parameters. Design-based stereology provides unbiased estimators of the parameters of interest by using a well-defined random sampling design, which requires no statistical model of the structure.

Suppose our aim is to estimate a geometrical parameter $Q(Y)$, such as volume, surface area, curve length, or particle number, for a finite object Y contained in three-dimensional space. Stereological estimators of $Q(Y)$ are stochastic versions of exact geometric identities of the form

$$Q(Y) = \int \alpha(Y \cap T)\, dT, \tag{1}$$

where $\alpha(Y \cap T)$ is a measure of the intersection between Y and a particular type of probe T. The integration in equation (1) ranges over all possible positions of the probe T and $\mathrm{d}T$ is the element of measure of T, which is invariant with respect to the group of translations and rotations (Santaló, 1976). Examples of $\alpha(Y \cap T)$ are the number of intersections between a surface and a test system of lines with an isotropic orientation and uniform random (UR) position in three dimensions, and the number of points of a UR positioned regular point grid intersecting the feature of interest. UR sampling (together with isotropic orientation in some cases) guarantees unbiasedness at the level of geometric sampling, but there are many ways to implement UR sampling, and some ways will be more precise than others. In particular, systematic uniform random (SUR) sampling often offers an appropriate balance between the estimator accuracy and the time spent ("cost").

2.2 *Parameter of Interest, Sampling, and Estimation*

Our parameter of interest is the total canopy surface area,

$$Q = \sum_{i=1}^{N_p} A'_i = \sum_{i=1}^{N_p} \sum_{j=1}^{M_i} A_{ij}, \tag{2}$$

where N_p is the total number of plants making up the canopy, A'_i is the total area of the ith plant, M_i is the number of leaves on the ith plant, and A_{ij} is the area of the jth leaf on the ith plant. The nondestructive estimation procedure involves three levels of sampling as illustrated in Figure 1:

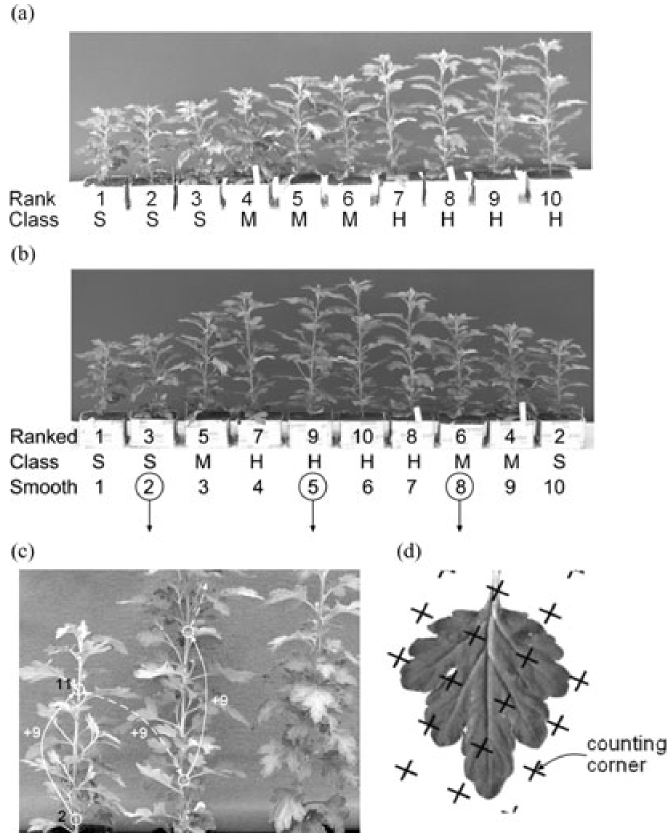

Figure 1. Three-stage sampling scheme. *Stage I*: (a) Plants are ranked in increasing size (leaf area), or an indicator of area such as height. Plants may also be assigned more roughly to a size category, e.g., S (short), M (medium), H (high), as shown. (b) Selection of sample of plants using the *smooth fractionator*. Plants are put in a symmetric arrangement by splitting the ranked plants (ranked order shown) into two similarly distributed parts in increasing and then decreasing size (smooth order indicated). A systematic UR sample (here with period $m_p = 3$) is then taken from the smooth arrangement. (c) *Stage II.* Selection of a systematic random sample of leaves from the sampled plants. The leaf insertion point defines the sampling unit (several leaves may emerge from an insertion point). Sampling units are ordered systematically from the bottom of the stem and upwards. If there are less than m_l insertion points remaining on a plant, the stepping procedure is continued on the next plant (counting from the bottom of the stem and upwards), which acts as the new "random start" (though dependent) for the next plant in the sample. (d) *Stage III.* Point counting to estimate area of leaves selected in the second sampling stage. A (0-dimensional) counting corner is specified (here, the upper right corner of the cross). The number of points hitting the leaf surface is multiplied by the grid constant a_p to obtain an estimate of the leaf area.

(i) SUR selection of plants from a canopy using the *smooth fractionator*, (ii) SUR selection of leaves from the sampled plants in (i) using the *fractionator* with a leaf insertion point defining a leaf sampling unit, and (iii) area estimation of the sampled leaves in (ii) using *point counting*. There can be more than one leaf in a leaf sampling unit (see Figure 1c), but for simplicity we will refer to a *leaf sampling unit* as a *leaf*.

We can define the estimator of Q associated with the first level of sampling as:

$$\hat{Q}^{(1)} = m_p \sum_{i \in S_1} A'_i, \tag{3}$$

where S_1 represents a random subset of evenly spaced integers with sampling period m_p from the set $\{1, 2, 3, \ldots, N_p\}$ and with a UR starting position within the set $\{1, 2, \ldots, m_p\}$. If large variations in surface area are visible between plants, then the "smooth" ordering procedure illustrated in Figure 1a and b is implemented and an SUR sample is taken from the reordered plants. This is an application of the smooth fractionator which aims to increase estimator precision by increasing within-sample variability and reducing the variability between samples (Gundersen et al., 1999; Gundersen, 2002).

When we take into account the second level of sampling, estimator (3) becomes:

$$\hat{Q}^{(2)} = m_p m_l \sum_{j \in S_2 \mid S_1} A_j, \tag{4}$$

where we have considered the sample of plants in equation (3) as a continuous system of leaves with areas $\{A_1, A_2, \ldots, A_{M(S_1)}\}$, where $M(S_1) = \sum_{i \in S_1} M_i$, and from which a systematic random sample of leaves with sampling fraction $1/m_l$ is extracted. In particular, S_2 represents a random subset of evenly spaced integers with sampling period m_l from the set $\{1, 2, \ldots, M(S_1)\}$ and with a UR starting position within the set $\{1, 2, \ldots, m_l\}$. Note that the random subset S_2 is a function of S_1, so that S_2 encapsulates the random nature of the two levels of sampling. Use of a sampling period proportional to any periodicity in the structure should be avoided. For example, chrysanthemum has a 5/8 phyllotaxis, i.e., the ninth leaf insertion point is positioned directly above the first, reached after three wounds around the stem.

The estimator of the total canopy surface area in equation (4) when leaf area is estimated using point counting can be expressed as:

$$\hat{Q}^{(3)} = m_p m_l \sum_{j \in S_2 | S_1} \hat{A}_j = m_p m_l a_p \sum_{j \in S_2 | S_1} P_j, \tag{5}$$

where the area of the *j*th leaf is estimated by superimposing, with a UR position, a square grid of points of area per test point a_p. If P_j is the number of points counted for the *j*th leaf, then an unbiased estimator of A_j is given by $\hat{A}_j = a_p P_j$. The result is a stochastic process where the observation of the phenomena is based on the repetition of the events (i.e., SUR positioned points) in the reference space (i.e., the canopy surface). Point counting is well established as being a highly efficient procedure to estimate the area of two-dimensional structures (e.g., Gundersen, Boysen, and Reith, 1981; Mathieu et al., 1981; Gundersen and Jensen, 1987). Other parameters can be readily estimated from the sample, such as the total number of leaves in the canopy, the mean leaf area per plant, etc.

2.3 *Variance Decomposition and Estimation*

The variance of the total area estimator $\hat{Q}^{(3)}$ can be decomposed into the following three terms:

$$\begin{aligned}\mathrm{Var}(\hat{Q}^{(3)}) = \mathrm{Var}(\hat{Q}^{(1)}) + \mathrm{E}\left[\mathrm{Var}\left(\hat{Q}^{(2)} \,\middle|\, S_1\right)\right] \\ + \mathrm{E}\left[\mathrm{Var}\left(\hat{Q}^{(3)} \,\middle|\, S_1, S_2\right)\right],\end{aligned} \tag{6}$$

where $\mathrm{Var}(\hat{Q}^{(1)})$ represents the variance of the area estimator due to between plants' area variability, $E[\mathrm{Var}(\hat{Q}^{(2)} \mid S_1)]$ is the mean variance due to between leaves' area over all possible realizations of S_1, and $E[\mathrm{Var}(\hat{Q}^{(3)} \mid S_1, S_2)]$ represents the mean variance (double expectation) of the area estimator due to point counting over all possible realizations of S_1 and S_2. For the derivation of equation (6) we have used the property of unbiasedness for estimators (4) and (5). Decomposition (6) can be used to express the square coefficient of error of the area estimator (5) (which is here defined as the variance of the area estimator divided by the square of the true area Q^2) in terms of the coefficients of error corresponding to each level of sampling. After dividing each side of equation (6) by Q^2, we have:

$$\mathrm{CE}^2(\hat{Q}^{(3)}) = \mathrm{CE}^2_{\text{plants}} + \mathrm{CE}^2_{\text{leaves}} + \mathrm{CE}^2_{\text{PC}}, \tag{7}$$

where we refer to each component of the coefficient of error (CE) of the total estimator as $\mathrm{CE}_{\text{plants}}$, $\mathrm{CE}_{\text{leaves}}$, and CE_{PC} for simplicity in the notation.

The coefficient of error of an estimator $\hat{Q}$ can be calculated based on k independent realizations of the estimator $\{\hat{Q}_1, \hat{Q}_2, \ldots, \hat{Q}_k\}$ by applying:

$$\mathrm{CE}^2(\hat{Q}) \approx \left(1 - \frac{1}{N}\right) \frac{1}{(k-1)} \frac{\sum_{i=1}^{k} (\hat{Q}_i - \bar{\hat{Q}})^2}{\left(\bar{\hat{Q}}\right)^2}, \tag{8}$$

where $\bar{\hat{Q}} = \frac{1}{k} \sum_{i=1}^{k} \hat{Q}_i$ and N is the number of all possible realizations of $\hat{Q}$. In practice this procedure can be prohibitively time consuming and methods to estimate variances from single samples are desirable. Devising suitable single sample variance predictors is generally very difficult because the observations of the data sample are not independent (e.g., Wolter, 1985; Mattfeldt, 1989; Cruz-Orive, 2004; Maletti and Wulfsohn, 2006). In the following sections, we will consider options for variance estimation. There are two situations of practical interest. One is to optimize allocation of effort between the three sampling stages based on a pilot study. Here, estimates of the individual terms on the right-hand side of equation (7) are needed. The second assumes that an optimized sampling design is being used, and an estimate of the total coefficient of error $\mathrm{CE}(\hat{Q}^{(3)})$ is required.

In this article, we apply equation (8) to calculate the first component on the right-hand side of equation (7) from k systematic sets of plants where a precise estimate of the area of each plant is available (see *smooth fractionator* approach in Section 2.2).

The estimation of the variance contribution due to point counting has been investigated in a large number of studies (e.g., Matheron, 1971; Matérn, 1985; Gundersen and Jensen, 1987). If the square grid of points is superimposed with isotropic orientation, then the variance of the area estimator $\hat{A}_j$ for the jth leaf can be approximated as follows:

$$\mathrm{Var}_{\mathrm{PC}}(\hat{A}_j) \approx 0.0724 u^3\, B(\partial Y_j), \tag{9}$$

where $u = a_p^{1/2}$ is the side length of the square grid test system and $B(\partial Y_j)$ is the boundary length of the leaf Y_j. If the superimposition of the grid on each leaf is done independently, then using the first identity of equation (5), we have:

$$\nu \equiv \mathrm{Var}_{\mathrm{PC}}\left(\hat{Q}^{(3)} \,\middle|\, S_1, S_2\right) \approx 0.0724 m_p^2 m_l^2\, u^3 \sum_{j \in S_2 | S_1} B(\partial Y_j), \tag{10}$$

and therefore, the double expectation of equation (10) over sigma algebras S_1, S_2 (which are produced by the first and second level of sampling, respectively) becomes:

$$\mathrm{E}\left[\mathrm{Var}_{\mathrm{PC}}\left(\hat{Q}^{(3)} \,\middle|\, S_1, S_2\right)\right] \approx 0.0724 m_p m_l u^3 B_T, \tag{11}$$

where B_T is the total boundary length of the canopy leaves. Dividing equation (11) by the square of canopy surface area $Q^2 = N_l^2 u^4 P_e^2$, where N_l is the total number of single leaves in the canopy and P_e is the mathematical expectation of the number of points counted per single leaf, the coefficient of error due to point counting can be approximated by:

$$\mathrm{CE}^2_{\mathrm{PC}} \approx 0.0724 \frac{B_e}{n_e u P_e^2} = 0.0724 \frac{B_e}{\sqrt{A_e}} \frac{1}{n_e P_e^{3/2}}, \tag{12}$$

where $n_e = N_l/(m_p \cdot m_l)$ is the expected number of (single) leaves in the sample, $B_e = B_T/N_l$ is the expected value of (single) leaf boundary, and $A_e = u^2 P_e$ is the expected value of (single) leaf area. An estimator of $\mathrm{CE}^2_{\mathrm{PC}}$ can be derived from equation (12), where P_e and n_e are estimated from the data sample, and the shape coefficient $B_e/\sqrt{A_e}$ is empirically estimated as described in Gundersen and Jensen (1987).

The problem of finding an estimator of $\mathrm{E}[\mathrm{Var}(\hat{Q}^{(2)} \,|\, S_1)]$ (second term on the right-hand side of equation 6) can be reduced to the problem of finding an estimator of $\mathrm{Var}(\hat{Q}^{(2)} \,|\, S_1)$ (i.e., of the variance of $\hat{Q}^{(2)}$ given a systematic sample of plants or a realization of S_1). Given S_1, we consider the sample of plants as a continuous system of leaves with areas A_i; $i = 1, 2, \ldots, M(S_1)$. The target parameter $Q^{(2)} \,|\, S_1$ can then be expressed as the integral of a step function $f(x) = m_p A_i$; $x \in [i-1, i)$, $i = 1, 2, \ldots, M(S_1)$ and $\hat{Q}^{(2)} \,|\, S_1$ can be interpreted as an unbiased estimator of $\int_0^{M(S_1)} f(x)\,\mathrm{d}x$ based on a systematic random sample of f with sampling period m_l (also known as a *Cavalieri* sample). The prediction of the variance of $\hat{Q}^{(2)} \,|\, S_1$ from a single systematic sample $\{f_1, f_2, \ldots, f_n\}$ of f has been widely addressed in the literature. In particular, several estimators have been proposed motivated by the problem of estimating the volume of a structure from a systematic sample of area sections (e.g., Matheron, 1971; Gundersen and Jensen, 1987; Cruz-Orive, 1989; Kiêu, 1997, Gundersen et al., 1999; García-Fiñana and Cruz-Orive, 2004). An estimator of the variance of $\hat{Q}^{(2)} \,|\, S_1$ when f is a piecewise continuously differentiable function with a finite set of discontinuities, can be expressed as follows:

$$\mathrm{var}\left(\hat{Q}^{(2)} \,\middle|\, S_1\right) = \frac{1}{12}\left(3C_0 - 4C_1 + C_2\right), \tag{13}$$

where $C_k = m_l^2 \sum_{i=1}^{n-k} f_i f_{i+k}$; $k = 0, 1, 2$. When the leaf area (leaf sampling unit) is not calculated directly, but estimated using point counting (i.e., $\hat{f}_i = m_p a_p P_i$; $i = 1, 2, \ldots, n$, where P_i is now defined as the number of points counted on the ith-leaf sampling unit of the sample), the variance estimator given in equation (13) needs to be corrected to take into account the random error associated with the estimation of f_i. The estimator of the square coefficient of error based on equation (13) becomes (see e.g., Kiêu, 1997):

$$\mathrm{ce}^2_{\mathrm{leaves}} = \frac{1}{\left(m_p m_l u^2 p_T\right)^2} \frac{1}{12}\left(3\left(C_0 - \hat{\nu}\right) - 4C_1 + C_2\right), \tag{14}$$

where p_T is the total number of points of the sample. The estimator of the variance component due to point counting is $\hat{\nu} = 0.0724 m_p^2 m_l^2 u^4 (\hat{B}_e/\sqrt{\hat{A}_e}) n^{1/2} p_T^{1/2}$, where n is the number of (single) leaves in the sample (note that this expression for $\hat{\nu}$ can be derived from equation (10)). An application of equation (14) for the volume estimator of brain compartments can be found in García-Fiñana et al., 2003).

We propose an alternative estimator of $\mathrm{CE}^2_{\mathrm{leaves}}$ based on k systematic subsamples from the set $\{f_1, f_2, \ldots, f_n\}$ (for a relatively similar approach, see Cruz-Orive, 1990). For simplicity in the notation, we refer to $\hat{Q}^{(2)} \,|\, S_1$ as $\tilde{Q}$, where $\tilde{Q} = m_l \sum_{i=1}^{n} f_i$ and we define the estimator $\tilde{Q}_k = k m_l \sum_{j=0}^{n_i - 1} f_{i+jk}$, where i is a UR integer in $[1, k]$ and n_i is the size of the corresponding systematic subsample (i.e., n_i is the largest integer not greater than $1 + (n + 1 - i)/k$). When point counting is applied to estimate the area of the sampled leaves, the two estimators become $\hat{Q} = m_l \sum_{i=1}^{n} \hat{f}_i$ and $\hat{Q}_k = k m_l \sum_{j=0}^{n_i-1} \hat{f}_{i+jk}$. Then, the coefficient of error of $\tilde{Q}$ can be estimated by applying:

$$\mathrm{ce}^2_{\mathrm{leaves}} = \frac{(\hat{Q})^{-2}}{k(k+1)}\left[\frac{1}{k-1}\sum_{i=1}^{k}\left(\hat{Q}_k^{(i)} - \hat{Q}\right)^2 - k\hat{\nu}\right]. \tag{15}$$

where $\{\hat{Q}_k^{(1)}, \hat{Q}_k^{(2)}, \ldots, \hat{Q}_k^{(k)}\}$ is the set of possible realizations of $\hat{Q}_k$ given $\{f_1, f_2, \ldots, f_n\}^n$.

In order to prove equation (15), we apply the variance decompositions: (i) $\mathrm{Var}(\hat{Q}) = \mathrm{Var}(\tilde{Q}) + \nu$ and (ii) $\mathrm{Var}(\hat{Q}_k) = \mathrm{Var}(\tilde{Q}_k) + k\nu$, where ν is the variance component of the estimator $\hat{Q}$ due to point counting (see definition in equation (10)) and where we have considered that the superimposition of the grid on each leaf is done independently. On the other hand, because f is a step function with a finite set of discontinuities, the variance of $\tilde{Q}$ for n relatively large can be approximated as (iii) $\mathrm{Var}(\tilde{Q}) \approx \frac{1}{k^2}\mathrm{Var}(\tilde{Q}_k)$ (e.g., Kiêu, 1997; Cruz-Orive, 2004; García-Fiñana and Cruz-Orive, 2004). Therefore, based on results (ii) and (iii), we have:

$$\mathrm{Var}(\hat{Q}_k) \approx k^2 \mathrm{Var}(\tilde{Q}) + k\nu. \tag{16}$$

Furthermore, the term $\mathrm{Var}(\hat{Q}_k)$ admits the following variance decomposition:

$$\mathrm{Var}(\hat{Q}_k) = \mathrm{E}[\mathrm{Var}(\hat{Q}_k \,|\, \hat{Q})] + \mathrm{Var}(\mathrm{E}[\hat{Q}_k \,|\, \hat{Q}]). \tag{17}$$

And after substituting equation (16) in equation (17), and using $\mathrm{E}[\hat{Q}_k \,|\, \hat{Q}] = \hat{Q}$ in combination with (i), we can write:

$$\mathrm{Var}(\tilde{Q}) \approx \frac{1}{(k^2-1)}[\mathrm{E}[\mathrm{Var}(\hat{Q}_k \,|\, \hat{Q})] - (k-1)\nu]. \tag{18}$$

After dividing equation (18) by $\hat{Q}^2$, and taking (i) into account and that an unbiased estimator of $\mathrm{E}[\mathrm{Var}(\hat{Q}_k \mid \hat{Q})]$ is given by $\frac{1}{k}\sum_{i=1}^{k}(\hat{Q}_k^{(i)} - \hat{Q})^2$, result (15) follows.

3. Experimental Program

The performance of the area estimator was evaluated with chrysanthemum (*Chrysanthemum morifolium* L. var. "Coral Charm"). The experimental plants were produced at the University of Copenhagen greenhouses. Cuttings were rooted in Grodan Delta blocks (10 × 10 × 6.5 cm) with grooved bases to promote drainage. The rooting process proceeded in a greenhouse propagation area for 20 days: the first 2 weeks under white plastic and the third one without cover.

Several experiments were carried out: (1) The precision of the point counting estimator was compared with that of several conventional 2D leaf area measurement methods widely used in botanical and horticultural research. (2) The contribution of the first and second levels of sampling to the overall variance of the surface area estimator was empirically calculated based on resampling from a large data set. We also applied the variance estimators derived from equations (12), (14), and (15) using single systematic samples to check their performance. (3) A growth chamber experiment was conducted to develop growth curves of chrysanthemum canopy surface area using the proposed protocol with "optimal" sampling parameters as determined from the previous experiments.

3.1 *Estimation of 2D Leaf Area*

The surface areas of ten "Coral Charm" chrysanthemum plants were measured exhaustively. All leaves were removed from the plants and leaf areas were measured using four different methods: (i) a commercial leaf area meter, (ii) digital image analysis, (iii) a leaf area model (LW), and (iv) point counting. The first two methods are destructive to the plant, whereas the latter two can be used nondestructively:

(1) The Li-3000C leaf area meter (LiCor Biosciences, Lincoln, Nebraska, U.S.A.) is an instrument designed for laboratories and is widely used as reference instrument. The sample is placed on a transparent conveyor belt and passes by a fluorescent light source. A projected image (shadow) is reflected by a system of three mirrors to a scanning camera. The resolution is on the order of 1 mm^2 and the reported coefficient of error as a function of sample area (in parentheses) is 2% (10 cm^2); 3% (3 cm^2); 6% (1 cm^2); and 10% (0.3 cm^2).

(2) Leaf profile images were taken using a 3CCD video camera (Model DXC-930P, Sony, Tokyo, Japan). Images were digitized, calibrated, segmented manually, and then individual areas computed using the BLOB routine in Matrox Inspector software (Matrox Electronic Systems, Dorval, Canada). The spatial resolution was 0.085 mm^2/pixel.

(3) The empirical "LW model" used in this work was developed by Jensen et al. (2006) for *Chrysanthemum morifolium* L. Leaf area (cm^2) was estimated from measurements of lengths and widths of leaf lamina using $\hat{a}_m = 0.62(l \cdot w) - 0.55$, where l is the length of lamina (cm) and w is the width (cm).

(4) Stereological point counting was repeated using three different grid intensities: 1.76, 4.32, and 8.12 cm^2/point. Grids were printed onto transparent film and randomly superimposed on the leaf profiles. Measurements were carried out only once for each grid. We estimated the shape factor, $B_e/\sqrt{A_e}$ in equation (12) to be 9 for "Coral Charm" leaves.

3.2 *Selection of Plants Using the Smooth Fractionator*

Fifty "Coral Charm" chrysanthemum plants were used to empirically calculate the contribution to the estimator variance due to taking a systematic sample of plants and to illustrate the increase in efficiency by using smoothing. The precision of several plant sampling designs were compared using sampling fractions $1/m_p = 1/5$, $1/10$, and $1/15$. The sampling designs were as follows:

(1) Smooth fractionator applied to the 50 plants ranked and ordered on the basis of the measured surface areas (Figure 2a and b). For each sampling fraction, all possible m_p area estimates based on m_p possible random starting points were obtained and $\mathrm{CE}^2(\hat{Q}^{(1)})$ was calculated by applying equation (8) with $N = k = m_p$.

(2) The 50 plants were each classified into one of the three size categories based on visual assessment of height: short (S), medium (M), and high (H), and randomized within the height groups before applying the smooth fractionator (Figure 2c). All possible samples were used to compute $\mathrm{CE}^2(\hat{Q}^{(1)})$ by applying equation (8) with $N = k = m_p$. This experiment was repeated three times with a new arrangement of plants each time, and the mean coefficient of error was computed.

(3) Simple random sampling without replacement of sample sizes $n_p = 10$, 5, and 4 or 3 (average 3.3) plants, to correspond to the above sampling fractions. The $\mathrm{CE}^2(\hat{Q}^{(1)})$ was computed by applying equation (8) with $k = 1000$ samples (where $1/N$ can be neglected). Alternatively, for cases $n_p = 10$ and 5, $\mathrm{CE}^2(\hat{Q}^{(1)})$ can be directly calculated by applying $(1 - 1/m_p)\mathrm{Var}(A^*)/(\bar{A}^{*2} n_p)$, where $\mathrm{Var}(A^*)$ is the variance between the 50 plant areas, $m_p = 50/n_p$ and $\bar{A}^*$ is the mean plant area.

3.3 *Fractionator Sampling of Leaves*

The contribution to the variance estimator due to systematic sampling of leaves was investigated using resampling from a large data set. Ten chrysanthemum plants (the same plants as in Section 3.1) were used to make up the canopy, and were considered as a continuous system of leaves. Sampling fractions of $1/m_l = 1/2$, 1/3, 1/6, 1/9, 1/12, 1/15, and 1/23 were used. All possible leaf samples were obtained (arising from all possible "random starts"). The experiment was repeated using the three point counting grids.

For each sampling fraction and grid intensity, the variance due to the subsampling leaves was estimated using equations (14) and (15), and was compared with the variance derived by resampling leaf sampling areas estimated using image analysis (considered as the reference method).

3.4 *Growth Chamber Experiment*

"Coral Charm" chrysanthemum cuttings were selected after a 20-day rooting period. Plants were grown in climate

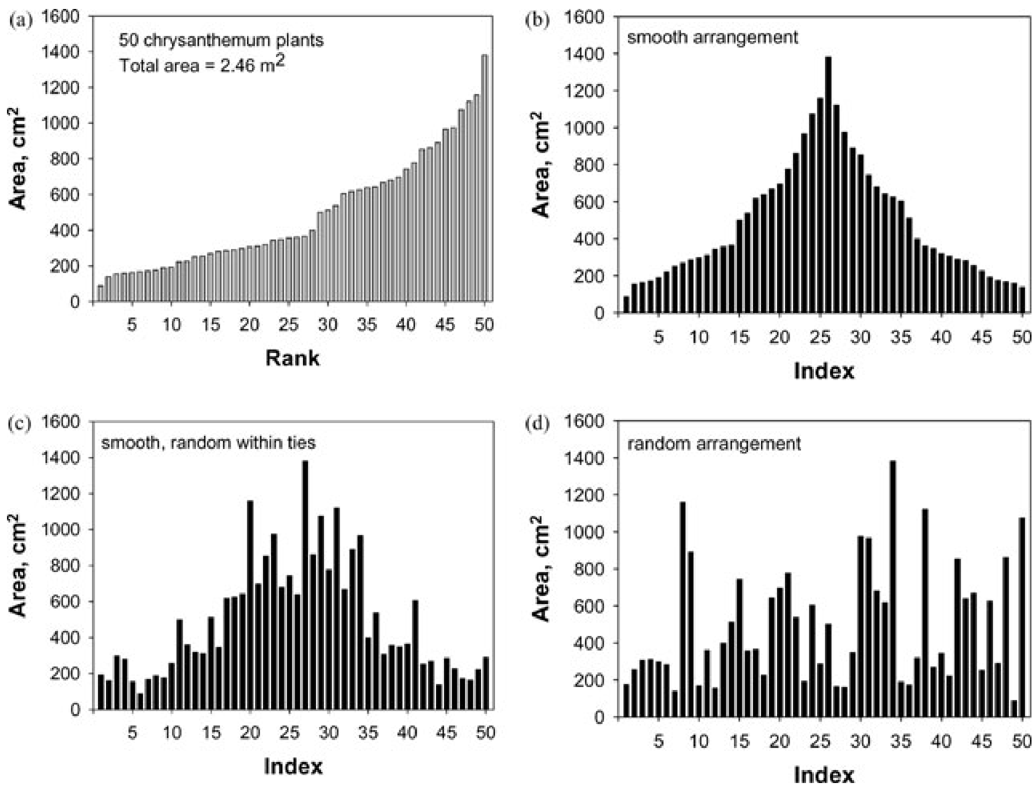

Figure 2. Different arrangements of 50 chrysanthemum plants, used to illustrate the effect of smoothing on the precision of SUR sampling at the first sampling stage. (a) Plants ranked in order of increasing surface area. (b) Plants in smooth arrangement based on surface area. (c) An example of an arrangement obtained by assigning each plant to a height category (S, M, H), randomizing plants within size groups, and then smoothing based on the three height groups. (d) An example of a simple random arrangement of the plants.

chambers under two different temperature regimes (treatments) with three replications. Each plant canopy was made up of 35 plants. The temperature regimes were selected to ensure difference in plant structure, especially height growth. Climate A was a positive DIF (difference between day and night temperatures) climate with a 25°C daytime temperature (16 hours) and 10°C nighttime temperature (8 hours). Climate B was a negative DIF climate with 19°C daytime temperature and 22°C nighttime temperature. Both treatments provided a 24-hour mean temperature of 20°C. Further details of the chambers and the climatic and lighting conditions are given in Jensen et al. (2006) and Sciortino (2005). The positive DIF climate normally results in shorter plants with smaller internodes while the negative DIF climate results in taller plants with larger internodes. The canopy was sampled using the described nondestructive stereological procedure (Figure 1) with sampling periods of $m_p = 5$, and $m_l = 4$, 3, or 1 (depending on plant age) and using a point counting grid of area per test point $a_p = 4.3$ cm^2. For the first sampling step, the plants were labeled following a smooth arrangement of their heights (in three height categories), based on visual inspection through the transparent chamber walls, and then systematically sampled. The plants in the sample were removed one by one from the chamber for further subsampling and measurement, and then immediately returned to the chamber, in order to remove the plants from the climate boxes for as short a time as possible. Canopy surface area was measured at the start of the experiment (day 0) and then on days 7, 14, and 21.

Data analysis and simulations were carried out using the `R` programming language (`R` Development Core Team, 2007).

4. Results

4.1 *Leaf Area Measurements*

The total plant leaf areas for 10 chrysanthemum plants are presented in Table 1 (the areas of all leaves were measured). The Licor proved the fastest procedure (5 minutes per plant) because of the use of a conveyer belt and in-built algorithms, but it is destructive and therefore not suited for in vivo studies of plant growth. Image analysis was used as the reference method to analyze the accuracy of the other leaf area estimators, but for this purpose leaves had to be removed from the plants. The leaf area empirical model was the most time consuming (1 hour per plant). Point counting took between 10–17 minutes per plant, depending on the grid density.

Similar estimates were given by image analysis and the finest point counting grid ($a_p = 1.76$ cm^2/point). The Licor and point counting with 4.32 cm^2 grid performed similarly. Because of the UR property of point counting, all grids provide unbiased estimators. However, each experiment produced only one estimate for that grid size, so that the individual and total plant areas will deviate from the true area. The leaf area

Table 1

Leaf areas of 10 "Coral Charm" chrysanthemum plants, as determined using different area estimation methods and a leaf sampling fraction of 1

Plant no.	No. leaves (no. sampling units)	Estimated total area cm² – Image analysis	LI-3000C	Point counting a_p 1.76 cm²	4.32 cm²	8.12 cm²	LW leaf model
1	51 (21)	781	732	803	766	641	732
2	58 (25)	925	872	935	900	885	925
3	64 (21)	1049	1094	1058	950	1088	989
4	76 (22)	1125	1069	1128	1073	1128	1008
5	72 (21)	1175	1129	1190	1134	1186	1055
6	71 (25)	1265	1249	1302	1268	1234	1270
7	65 (33)	1116	1092	1128	1103	1088	1055
8	77 (23)	1071	981	1081	1069	1048	985
9	61 (22)	981	987	1003	1060	1007	903
10	67 (22)	897	914	891	904	861	819
Sum	662 (235)	10,385	10,119	10,519	10,227	10,166	9741
Mean	66 (23)	1038	1012	1052	1022	1016	974
$\bar{A}^{-1}\sqrt{\frac{1}{10}\sum_{i=1}^{10}\Delta_i^2}$			4.5%	1.7%	4.5%	5.0%	7.3%
$\bar{P}_T$				598	237	123	

Note: $\Delta_i = \hat{A}_i - A_i$, where A_i and $\hat{A}_i$ are the ith plant area obtained by image analysis (reference value) and point counting (or the LW leaf model or area meter), respectively. $\bar{A}$ is the mean area per plant obtained from the image analysis and $\bar{P}_T$ = mean point count per plant.

Table 2

Estimated CE_{plants} of the estimated total area of 50 chrysanthemum plants, for different arrangements of the plants (see Figure 2)

CE_{plants}	Sample size (Sampling fraction) 10 (1/5)	5 (1/10)	3–4 (1/15)
SUR of smooth arrangement (by area)	0.021	0.076	0.077
SUR of smoothed, random within height class groups	0.104	0.184	0.203
Simple random arrangement	0.205	0.276	0.335

empirical model underestimated plant areas with 7% error on average.

4.2 *Performance of the Smooth Fractionator*

The effect of smoothing on the precision of the canopy area estimator when sampling at the plant level is summarized in Table 2. Smoothing according to the areas resulted in a significant improvement in the precision of the area estimator. With a sample size of 10 the CE of the area estimator error was about 2%, while for three to four plants it was 8%. In comparison, selecting plants using simple random sampling increased the CE by factors of about 10 and 5, respectively. Ranking the plants into three size classes reduced the power of smoothing, but still provided considerably lower variances compared to simple random sampling.

4.3 *Performance of Variance Prediction Models*

Table 3 shows the empirical coefficient of error of the canopy area estimator due to the second component on the right-hand side of equation (7) for different sampling fractions. As the number of leaves being sampled n increases, CE_{leaves} decreases approximately as n^{-1}. A sample area of about 0.1 m² (leaf sampling fraction of about 1/12) resulted in $CE_{leaves} \approx 7\%$. When the contribution due to point counting is added, then the CEs are approximately 7.3%, 8.1%, and 9.6% for point grid sizes equal to 1.76, 4.32, and 8.12 cm²/point, respectively (these correspond to about 600, 240, and 130 points per plant). The contribution due to point counting decreases as $n^{-1/2}$ as one would expect based on equation (12). The predictions of the CE using estimator (14) in combination with equation (12) are reasonably close to the empirical values (the values refer to the square root of the average over all the possible estimates of the CE² based on the whole data set). For small sample sizes (e.g., $m_l = 23$), the estimator performance is poor and this could be explained by the fact that equation (14) is based on asymptotic approximations of the variance for n large (Matheron, 1971; Kiêu, 1997; García-Fiñana and Cruz-Orive, 2004). The prediction based on equation (15) is somewhat unstable for $m_l \geq 12$.

4.4 *Canopy Surface Area Development*

Figure 3 shows the development of total leaf area of canopies made up of 35 "Coral Charm" *chrysanthemum* plants grown in transparent climate chambers, under two temperature regimes. We did not carry out an experiment to estimate CE_{plants} for these experiments. The between plant variability within a growth chamber at any time was smaller than for

Table 3

Empirical coefficients of error (%) of the canopy area estimator (stages II-III) for different leaf sampling periods

		Point counting grid											
	(%)	1.76 cm^2				4.32 cm^2				8.12 cm^2			
m_l	CE_{leaves}	CE_{PC}	$CE_{leaves+PC}$	$ce^{Cav}_{leaves+PC}$	$ce^{Sub}_{leaves+PC}$	CE_{PC}	$CE_{leaves+PC}$	$ce^{Cav}_{leaves+PC}$	$ce^{Sub}_{leaves+PC}$	CE_{PC}	$CE_{leaves+PC}$	s $ce^{Cav}_{leaves+PC}$	$ce^{Sub}_{leaves+PC}$
2	0.7	0.9	1.1	1.5	1.5	1.7	1.8	2.1	1.5	2.8	2.8	2.9	2.6
3	1.1	1.0	1.5	2.2	2.8	2.0	2.3	2.9	3.5	3.4	3.5	3.6	3.3
6	4.5	1.5	4.7	3.6	3.2	2.9	5.3	4.5	3.5	4.8	6.5	5.6	5.6
9	5.1	1.8	5.4	5.8	4.7	3.6	6.2	6.8	5.9	5.9	7.7	7.6	6.2
12	7.0	2.1	7.3	6.8	11.1	4.2	8.1	7.9	12.3	6.8	9.6	8.9	11.7
15	6.7	2.3	7.1	7.1	5.2	4.7	8.1	8.4	6.5	7.6	9.9	9.5	7.8
23	13.4	2.9	13.8	7.4	6.1	5.7	14.6	9.1	8.3	9.4	16.2	11.0	10.3

Note: CE_{leaves} (second component on the right-hand side of equation (7)) was empirically estimated from subsamples of the area data set acquired using image analysis. CE_{PC} is the term due to point counting (third level of sampling) calculated for each of three grid sizes using equation (12). The contribution from both terms CE_{leaves} and CE_{PC} was obtained by applying the expression $CE_{leaves+PC} = (CE^2_{leaves} + CE^2_{PC})^{1/2}$. Estimates of $CE_{leaves+PC}$ were obtained by applying (i) equations (14) and (12) ($ce^{Cav}_{leaves+PC}$), and (ii) equations (15) and (12) ($ce^{Sub}_{leaves+PC}$).

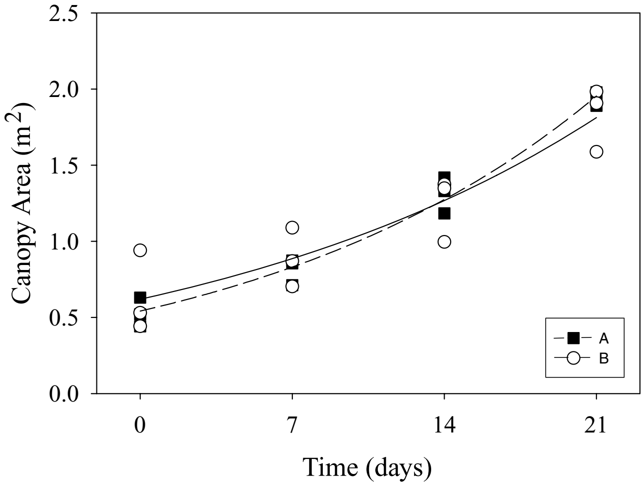

Figure 3. Growth of canopies (35 chrysanthemum plants) under two different temperature regimes, estimated using the described nondestructive stereological procedure with $m_p = 5$ plant sampling period (smooth), $m_l = 1$, 1, 3, or 4 leaf sampling period (at 0, 7, 14, and 21 days, respectively), and $a_p = 4.3$ cm^2 point counting grid.

the experiments described in previous sections because plants were of the same age. Analysis based on mixed effects modeling indicated that canopy area increased significantly over time at a rate of 675 cm^2 day^{-1} (p-value < 0.001) for treatment A, and 560 cm^2 day^{-1} for treatment B ($p < 0.001$). However, the change over time does not differ between treatments ($p = 0.109$). Treatment B plants had noticeably smaller leaf internode lengths and consequently a more compact canopy, as expected.

5. Discussion

We developed and tested a practical, nondestructive methodology for canopy leaf area estimation and prediction of the estimator variance. The procedure consists of three sampling stages: (i) SUR sampling of plants, (ii) SUR sampling of leaves, and (iii) estimation of sampled leaf area using point counting. As plants grow larger or with more complex branching, further fractionator subsampling steps can be introduced, e.g., subsampling of stems within branches (e.g., Wulfsohn, Maletti, and Toldam-Andersen, 2006), and of leaflets within leaves. Because the leaves represent a uniform sample of the canopy it is also possible to estimate other attributes of the canopy from the sample, such as distribution of surface area with height and leaf orientation distributions.

Even with the very precise image analysis, biases may be introduced due to the image segmentation operation as well as due to folded leaves and the natural overlapping of leaf lobes (overlapping can be detected and partially corrected for, c.f. Warren, 2000). Biases due to folds and overlapping are also introduced using the Licor area meter. Manual point counting readily avoids this error; every time that a point hits a folded or overlapped part it can be counted twice. The bias of the "LW model" highlights the need to calibrate empirical models for plants grown under different conditions.

The precision of the overall sampling design depends on the spatial homogeneity of leaf areas across the canopy (between plants and between leaves along the stem); any random variation in the size of the sample at each stage; leaf shape; and the number of points that are counted (or the sample area). The use of several nested sampling stages permits the sampling effort to be distributed across the canopy, while still achieving sufficient sample sizes to attain the desired precision. The purpose here should be to assign more sampling effort at stages where the variation is larger. A pilot study can be used to determine sampling allocation effort. Based on Table 2, our recommendation is that if a sample size of about 10 chrysanthemum plants is used and the time and effort is made to carefully order plants by size prior to smoothing, then the variance component due to sampling of plants can be considered negligible. If the smooth fractionator is applied using size classes then the variance due to taking a sample of plants should be estimated at least once for a given groups of plants, e.g., using equation (8) with $k = 2$ independent replicates and either a precise estimate of leaf area or combined with equations (14) and (12).

6. Conclusion

A stereological procedure was developed for nondestructive estimation of quantitative parameters associated with canopy structure, including total surface area and number of leaves, and other derived quantities. Several subsampling stages were used to provide a methodology that is efficient and straightforward to execute, while yielding unbiased estimators. Where there is visible between-plant variability, *smoothing* of plants by size before systematic sampling can be used to reduce the component of sampling error variance arising from the selection of a sample of plants.

Point counting provided unbiased estimators of plant and total canopy surface areas from sparse samples with precisions as good as or better than those provided by sophisticated and expensive equipment. It also allowed for nondestructive measurements. For mature "Coral Charm" chrysanthemum plants, a leaf sampling period of 9 followed by point counting using a 4.3 cm^2/point grid yielded total canopy area estimates with a CE of less than 7%.

A combination of empirical resampling and estimators based on asymptotic approximations were proposed to predict the variance of the area estimator for a given set of plants. The use of the "Cavalieri" variance estimator approach applied at the leaf sampling stage combined with the point counting variance estimator provided good predictions.

Acknowledgements

This research was carried out while MS was visiting the Faculty of Life Sciences, University of Copenhagen (previously the Royal Veterinary and Agricultural University, Denmark) from Bologna, Italy during his Ph.D. studies. The authors acknowledge funding from SJVF (Denmark) project "Applications of Stereology in Agricultural Systems," from the Spanish Ministry of Education and Sciences I+D Project MTM2005-08689-C02-01, and from the Royal Society Joint Project 2008/R1. Thanks to Hans-Jørgen Gundersen for valuable discussions and to Guido Baldoni. We are grateful to Mikaela Vasstrøm and Andrea Andreassen for experimental assistance, and to Alan Hansen for technical support. We thank an anonymous reviewer for comments that led to substantial improvements of the article.

References

Baddeley, A. and Jensen, E. B. V. (2004). *Stereology for Statisticians*. Boca Raton, Florida: Chapman and Hall/CRC.

Baker, B., Olszyk, D. M., and Tingey, D. (1996). Digital image analysis to estimate leaf area. *Journal of Plant Physiology* **148,** 530–536.

Bignami, C. and Rossini F. (1996). Image analysis estimation of leaf area index and plant size of young hazelnut plants. *Journal of Horticultural Science* **71,** 113–121.

Bréda, N. J. J. (2003). Ground-based measurements of leaf area index: A review of methods, instruments and current controversies. *Journal of Experimental Botany* **54,** 2403–2417.

Cruz-Orive, L. M. (1989). On the precision of systematic sampling: A review of Matheron's transitive methods. *Journal of Microscopy* **153,** 315–333.

Cruz-Orive, L. M. (1990). On the empirical variance of a fractionator estimate. *Journal of Microscopy* **160,** 89–95.

Cruz-Orive, L. M. (2004). Precision of the fractionator from Cavalieri designs. *Journal of Microscopy* **213,** 205–211.

García-Fiñana, M. and Cruz-Orive, L. M. (2004). Improved variance prediction for systematic sampling on R. *Statistics* **38,** 243–272.

García-Fiñana, M., Cruz-Orive, L. M., Mackay, C. E., Pakkenberg, B., and Roberts, N. (2003) Comparison of MR imaging against physical sectioning to estimate the volume of human cerebral compartments. *NeuroImage* **18,** 505–516.

Gundersen, H. J. G. (2002). The smooth fractionator. *Journal of Microscopy* **207,** 191–210.

Gundersen, H. J. G. and Jensen, E. B. (1987). The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy* **147,** 229–263.

Gundersen, H. J. G., Boysen, M., and Reith, A. (1981). Comparison of semiautomatic digitizer-tablet and simple point counting performance in morphometry. *Virchows Archiv B Cell Pathology* **37,** 317–325.

Gundersen, H. J. G., Jensen, E. B. V., Kiêu, K., and Nielsen, J. (1999). The efficiency of systematic sampling in stereology—reconsidered. *Journal of Microscopy* **193,** 199–211.

Heinicke, D. R. (1966). The micro-climate of fruit trees. III. The effect of trees size on light penetration and leaf area in Red Delicious apple trees. *Proceedings of the American Society for Horticultural Science* **84,** 33–41.

Jensen, T. L., Rosenqvist, E., and Aaslyng, J. M. (2006). A daylight climate chamber designed for climatic control and calculation of canopy CO_2 exchange. *Hort Technology* **16,** 191–198.

Jonckheere, I., Fleck, S., Nackaerts, K., Muys, B., Coppin, P., Weiss, M., and Baret, F. (2004). Review of methods for in situ leaf area index determination. Part I. Theories, sensors and hemispherical photography. *Agricultural and Forest Meteorology* **121,** 19–35.

Kiêu. K. (1997). Three lectures on systematic geometric sampling, Vol. **13**. *Memoirs*. Department of Theoretical Statistics, University of Aarhus, Denmark.

Long, S. P. and Hällgren, J. E. (1993). Measurement of CO_2 assimilation by plants in the field and the laboratory. In *Photosynthesis and Production in a Changing Environment: A Field and Laboratory Manual*, D. O. Hall, J. M. O. Scurlock, H. R. Bolár-Nordenkampf, R. G. Leegood, and S. P. Long (eds), 129–167. Norwell, Massachusetts: Chapman and Hall.

Maletti, G. M. and Wulfsohn, D. (2006). Evaluation of variance models for fractionator sampling of trees. *Journal of Microscopy* **222,** 228–241.

Matheron, G. (1971). *The Theory of Regionalized Variables and Its Applications*. Les Cahiers du Centre de Morphologie Mathématique de Fointainebleau, No.5. École Nationale Suipèrieure des Mines Paris.

Mathieu, O., Cruz-Orive, L. M., Hoppeler, H., and Weibel, E. R. (1981). Measuring error and sampling variation in stereology: Comparison of the efficiency of various methods for planar image analysis. *Journal of Microscopy* **221,** 75–88.

Matérn, B. (1985). Estimating area by dot counts. In *Contributions to Probability and Statistics in Honour of Gunnar Blom*, J. Lanke and G. Lindgren (eds), 243–257. Lund, Sweden: University of Lund.

Mattfeldt, T. (1989). The accuracy of one-dimensional systematic sampling. *Journal of Microscopy* **153,** 301–333.

R Development Core Team. (2007). A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Radtke, P. J. and Bolstad, P. V. (2001). Laser point-quadrat sampling for estimating foliage-height profiles in broad-leaved forests. *Canadian Journal of Forestry Research* **31,** 410–418.

Ray, R. C. and Singh, R. P. (1989). Leaf area estimation in capsicum (*Capsicum Annuum* L.). *Scientia Horticulturae* **39,** 181–188.

Robbins, N. S. and Pharr, D. M. (1987). Leaf area prediction models for cucumber from linear measurements. *HortScience* **22,** 1264–1266.

Santaló L. A. (1976). Integral geometry and geometric probability. In *Encyclopedia of Mathematics and its Applications* Vol. **1**, G. -C. Rota (ed), Reading, Massachusetts: Addison-Wesley.

Sciortino, M. (2005). Innovative methods to study the influence of climatic parameters on canopy photosynthesis for agricultural purposes. Ph.D Thesis, University of Bologna, Italy.

Sellin, A. (2000). Estimating the needle area from geometric measurements: Application of different calculation methods to Norway Spruce. *Trees* **14,** 215–222.

Warren, D. (2000). Image analysis in chrysanthemum DUS testing. *Computers and Electronics in Agriculture* **25,** 213–220.

Warren Wilson, J. (1960). Inclined point quadrats. *New Phytologist* **59,** 1–8.

Warren Wilson, J. and Reeve, J. E. (1959). Analysis of the spatial distribution of foliage by two dimensional point quadrates. *New Phytologist* **58,** 92–101.

Watson, D. J. (1947). Comparative physiological studies on the growth of field crops: I. Variation in net assimilation rate and leaf area between species and varieties, and within and between years. *Annals of Botany* **11,** 41–76.

Welles, J. M. (1990). Some indirect methods of estimating canopy structure. *Remote Sensing Reviews* **5,** 31–43.

Wolter, K. (1985). *Introduction to Variance Estimation*. New York: Springer-Verlag.

Wulfsohn, D., Maletti, G. M., and Toldam-Andersen, T. B. (2006). Unbiased estimator for the total number of flowers on a tree. *Acta Horticulturae* **707,** 245–251.

Wünsche, J. N., Lakso, A. N., and Robinson, T. L. (1995). Comparison of four methods for estimating total light interception by apple trees of varying forms. *HortScience* **30,** 272–276.

Received November 2007. *Revised December* 2008.
Accepted December 2008.