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Short communication

Quantification of microsclerotia of *Verticillium dahliae* in plant material by image analysis

L. Mol[1] and E. M. J. Meijer[2]

[1] *Department of Agronomy, Wageningen Agricultural University, Haarweg 333, 6709 RZ Wageningen, The Netherlands;* [2] *Research Institute for Plant Protection-DLO, P.O. Box 9060, 6700 GW Wageningen, The Netherlands*

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Abstract

A procedure for the quantification of microsclerotia of *Verticillium dahliae* with an image analysis system was compared with counting by eye. Colonised potato plant material was used from plants grown in pathogen-free soil in a greenhouse and from twelve crops (including four potato cultivars) grown outdoors in pots filled with pathogen-free soil under natural conditions. The values obtained from the potato material from the greenhouse were comparable for both methods. Variation in the results mainly resulted from sampling errors. The numbers of microsclerotia in plants grown outdoors were overestimated by image analysis for most crops. The source of the error was related to the presence of plant and soil particles that did not discolour during boiling of the samples in sodium hydroxide. Image analysis was a suitable and reliable method for assessing the number of microsclerotia only in potato haulm samples from plants grown in pathogen-free soil in the greenhouse.

Abbreviations: cv = coefficient of variation; IA = image analysis; MS = microsclerotia, microsclerotium.

Verticillium dahliae Kleb. forms microsclerotia on senescing plant material. Direct estimations of the reproduction of *V. dahliae* on plant parts by estimating the area colonised with MS or by counting the MS seem unreliable for quantitative analysis. Problems are the very high numbers of MS that can be produced per unit plant material and the large differences within and among plant parts [Menzies, 1970].

Ben-Yephet and Szmulewich [1985] assessed the numbers of MS in potato debris by direct counting in small subsamples. Slattery [1981] and Davis *et al.* [1983] plated ground potato stem material on a semi-selective medium. Their method proved to be reliable, but they could not distinguish between colonies from single or aggregated MS in the debris, because grinding does not sufficiently disaggregate microsclerotia. Moreover, plating of plant debris is time and space consuming.

In this paper counting by image analysis (IA) is compared with counting by eye for its suitability to quantify the MS production in plant material grown in a greenhouse (Source 1) and outdoors (Source 2).

For Source 1, potato (*Solanum tuberosum*) plants (cvs Element and Mirka) were infested by immersing rooted sprouts in a blended culture suspension of *V. dahliae* before planting in pure quartz sand or potting compost. Plants were grown in the greenhouse and no other pathogens were found in the plants. Colonised plant material was collected from plants harvested 72 days after planting (green and immature) or 113 days after planting (stems were senescing and close to maturity).

For Source 2, 12 crops were grown in six replications: potato (*Solanum tuberosum* cvs Element, Mirka, Ostara and Astarte), pea (*Pisum sativum* cv.

Finale), sugar beet (*Beta vulgaris* cv. Univers), onion (*Allium* cepa cv. Jumbo), flax (*Linum usitatissimum* cv. Viking), spring barley (*Hordeum vulgare* cv. Prisma), field bean (*Vicia faba* cv. Victor), spring wheat (*Triticum aestivum* cv. Minaret), and spring rape (*Brassica napus* cv. Petranova). The crops were grown in 20-L pots filled with a mixture (1:1 by volume) of potting compost and clay soil in which never a crop was grown. Pots were placed in the open air under natural day length and temperature, and were wrapped in insulating foil to prevent heating of the pots by solar radiation. Plants were infested by mixing MS of *V. dahliae* with the soil. Aerial plant parts, stubble and roots were harvested and collected as described by Mol [1995]. After the harvest the plant material was air-dried.

For both sources, the air-dried plant material was dry ground with a mill accommodated with a 1 mm mesh sieve. Samples were bleached by boiling for 20 min in 25 mL 1.0 *M* NaOH. During bleaching almost all plant material discoloured, but MS remained black as well as some particles of the plant material. Two methods of counting were used: counting by eye and counting by IA.

For counting the MS by eye in the potato plant material from Source 1, the solution of a 20–25 mg bleached sample was filtrated over a Büchner-funnel with a radius of 50 mm. The filter paper with the sample was subsequently dried at room temperature and stored until counting. The MS in the material from the 12 crops from Source 2 were counted in the same bleached sample as used for counting by IA. Before counting, the filter paper with the sample was put on a 0.5 cm mesh grid, and rewetted. A stereo dissecting microscope (magnification 24×) and a hand counter were used to count the MS in the whole sample. A part of the sample was counted when the number of MS counted exceeded 1000.

A SUN-based IA system; GOP 302 (Context Vision Sweden) running under UNIX was used to count the MS automatically. MicroGOP (Context Vision Sweden) was used as a software package, containing most routines necessary for image processing, scan stage control and other level algorithm calls (C, UNIX-shells). After bleaching a 10–15 mg sample, the solution was filtered over a modified Büchner-funnel with an area of 22×22 mm^2. The filter paper with the sample was dried at room temperature and then stored until counting. Samples were not rewetted before scanning because the contrast was better with dry samples. On a white plate of 24.5×24.5 cm^2, 49 samples were glued with water soluble glue in a 7×7 square pattern. Between two samples a free space was kept of 7 mm. This free space was necessary to scan the sample borders. The plate with the samples was put on an 8 inch scan stage table (Marzhauser), computer controlled by a programmable scan stage controller SSCO2 (IDUNA). The scan stage was part of a Stabiplan microscope (Leica) fitted on marble table and equipped with a M420 Macroscope (Wild). With the 1:5 macrozoom objective at position 18, images of 6 mm in diameter were obtained. A 2/3 inch CCD black and white camera (Fujitsu) was used with a resolution of 582×500 pixels plus a 0.4 inch photo-tube to attach it to the M420. An additional cold-light source KL15000 (Scott) with a glass fibre ring illuminator was used to obtain higher contrast and larger depth of field. The microscope magnification was a compromise between the required scan area and resolution; four images in both x- and y-directions were necessary to cover one sample completely. The quadrants were automatically positioned under the microscope by the scan stage which sequentially meandered through all the samples on the object carrier (white plate). Immediately after scanning the image was analysed by the programme to detect only those particles of the size of MS. This was done by means of a RANK (MAXMIN) filter which made little particles disappear in the image. The obtained image was subtracted from the original image so that the resulting image showed the sample without shading and with clear tiny spots. One series of 49 samples (i.e. 16 × 49 = 784 images) took nearly 5 h to scan and analyse. Because it was not necessary to be stand-by, the analysis ran mostly at night or at off-time. After analysis one could print out the number of MS per image, per sample or per plate.

Numbers of microsclerotia in samples from Source 1 obtained by the two counting methods, were compared by linear regression analyses. The results of the samples from Source 2 were analysed by analysis of variance. Pairwise comparisons between the counting methods were made by LSD.

The IA method gave reliable countings since counting twice a set of the same bleached subsamples of Source 1 resulted in equal values (R^2 = 0.99; Fig. 1). In samples from Source 1 obtained form immature or mature plants, IA counting agreed with counting by eye, when separate subsamples were taken for the two methods (R^2 = 0.93; cv = 16%; Fig. 2). A number of samples from Source 1 in a range of 0–1,000 MS per sample were subsampled twice and counted by IA

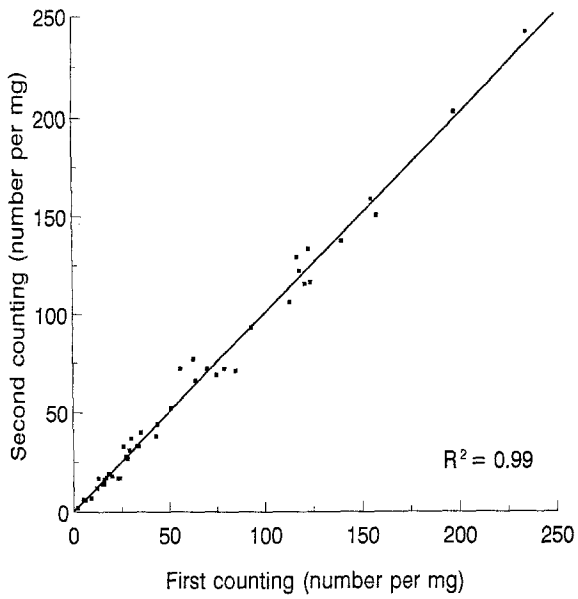

Fig. 1. Relation between the values obtained after counting the same series of prepared samples twice by image analysis. The line represents a 1:1 relationship. Source 1.

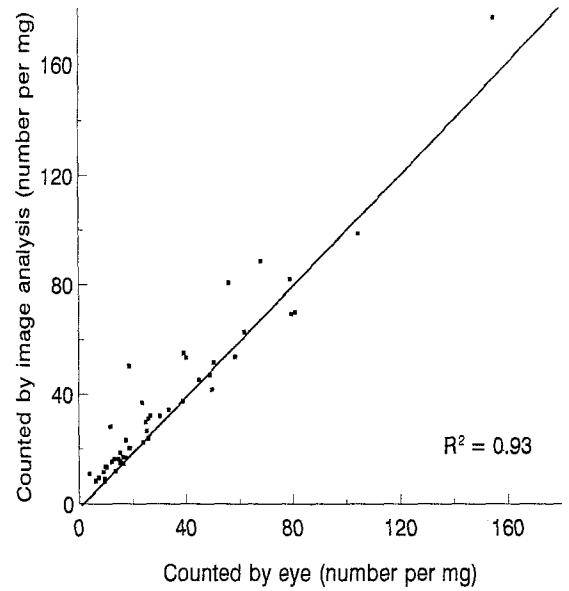

Fig. 2. Number of microsclerotia counted by eye related to the number counted image analysis. The line represents a 1:1 relationship. Source 1.

(Fig. 3). There was a high correlation between the first and second counting ($R^2 = 0.85$; cv = 17%). With an increasing MS density in the sample the standard deviation became higher, but the coefficient of variation remained the same over the whole range. The mean of all numbers counted in the first series of subsamples was very close to the mean of the numbers counted in the second subsample. After 13 times repeated subsampling of one source of plant material with a low (7×10^3 MS.g^{-1}) and one with an intermediate density (32×10^3 MS.g^{-1}), the coefficients of variation were 13% and 18%, respectively. So, a major part of the variation in the results of counting can be ascribed to sampling errors. When this sampling error is considered a lower coefficient of variation cannot be expected when counting by eye and by IA are compared. It can be concluded that determination of MS in plant material of potato was very accurate for plant material from Source 1.

In samples from Source 2, only for the aerial parts of potato cvs. Elements and Ostara, and barley a good correlation (albeit a small overestimation) existed between counting by eye and counting by IA, but for the other crops, counting by IA showed a significant over-estimation of the number of MS compared to the values obtained by eye (Table 1). For Source 1, plants were grown in quartz sand. This soil type is easy to

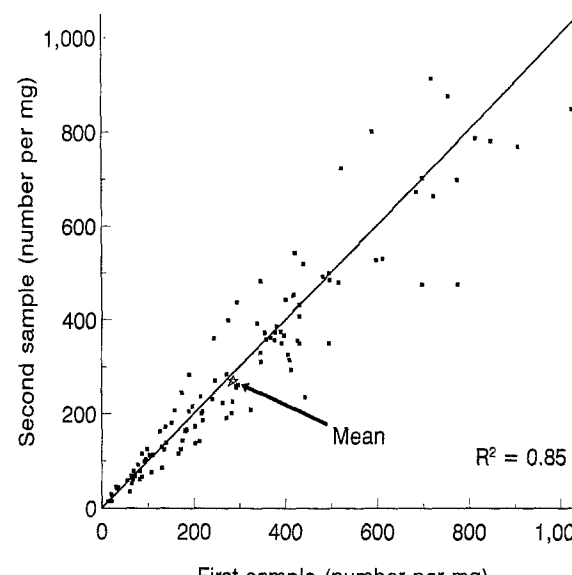

Fig. 3. Relation between the numbers of microsclerotia found in a first subsampling of a set of 123 potato plant material samples and the numbers of MS found in a second subsampling of the same set. The line represents a 1:1 relationship. Source 1.

separate from subterranean plant parts and did not turn dark after boiling in NaOH. For Source 2, plants were grown in a mixture of clay soil and potting compost. This soil was very difficult to remove from the sub-

Table 1. Number of microsclerotia per mg aerial plant material, stubble and root of 12 crops, counted by eye and by image analysis (IA). Source 2

Crop	Microsclerotia per mg					
	Aerial parts		Stubble		Root	
	Eye	IA	Eye	IA	Eye	IA
Potato 'Element'	131	158 ns	37	96 ***	9	49 **
Potato 'Ostara'	94	115 ns	34	61 ***	6	40 **
Potato 'Astarte'	47	86 ***	15	62 ***	3	23 ***
Potato 'Mirka'	34	76 ***	12	62 ***	6	66 **
Flax	26	34 ***	–[a]	–[a]	6	33 **
Field bean	4	43 ***	3	17 ***	2	36 ***
Pea	10	46 **	5	87 **	3	25 ***
Barley	14	20 ns	19	61 ***	5	59 ***
Onion	3	44 ***	–[a]	–[a]	4	24 **
Wheat	3	11 ***	5	53 ***	4	64 **
Sugar beet	4[b]	66 [b,] **	4c	146 [c,] ***	1	25 **
Rape	2	17 ***	4	23 ***	3	31 ***

*, **, ***, Results obtained with the image analysis system significantly differ from countings by eye at $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively; ns = not significant.

[a] No stubble left with the harvest methods used.

[b] Results are from dead leaves.

[c] Results are from leaves and epicotyl.

terranean parts. Soil particles did not always discolour after boiling with NaOH, whereas also the plant material itself did not always lose its colour. Presumably, these are the main reasons for the overestimation. The stubble (green leaves and epicotyl) of sugar beet turned dark after boiling. It is obvious that the high numbers in those samples are unrealistic.

In aerial material of the plant species tested that did not show difference between the methods also some non-MS particles were counted, but the number of MS was so high that this did not cause an unwarranted deviation. So, the use of the IA system is only possible if the relative number of dark particles in the sample after boiling other than MS of *V. dahliae* is (very) low and when the plant material discolours during boiling. Boiling samples longer or in a higher concentration of NaOH did not improve the results.

The advantage of counting MS automatically is most evident in samples with a very high density. Counting of those samples by eye is almost impossible, because MS are too close to each other. Then, subsampling and diluting is another possibility. This would not solve the problem of aggregates containing many MS and may increase the variation. To diminish the variation by the IA method, (sub)sample sizes should be increased. This is only possible when the surface of the filter paper, and as a consequence the surface scanned by IA are larger. To diminish the variation caused by subsampling it would be better to take more subsamples to one source. The number of subsamples will be a function of the accuracy required.

Selection on size is easily done by the computer programme and one of the possibilities to improve the results. Particles larger than MS of *V. dahliae* can be excluded (for example sclerotia of *Colletotrichum coccodes*). With the current IA method, it is impossible to discern between MS of *V. dahliae* and organisms which form structures with a similar size (for example MS of *V. tricorpus*). For that purpose other clearing techniques should be investigated. Specific staining could be a tool to overcome the problem with both the polluting non-biotic particles and other organisms. For now, the IA method should not be used for samples polluted with other dark particles, or should be used in combination with plating methods.

Because in potato material from Source 1 the correlation between IA counts and counts by eye was very good, for that purpose the IA method should be preferred. With IA the capacity is much higher and counting is less time consuming. Counting MS of *V. dahliae* by image analysis requires more research before it can be given a broader application.

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