Spore dimensions of *Puccinia* species of cereal hosts as determined by image analysis

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**Abstract:** Digital image analysis was used to measure dimensions of spores produced by *Puccinia coronata*, *P. graminis*, *P. hordei*, *P. recondita*, *P. striiformis* and *P. triticina*. Included were teliospores, basidiospores, urediniospores and, except for *P. striiformis*, pycniospores and aeciospores. Length, width and projection area of spores were measured with NIH Image or Scion software. By using limits on size, spores were automatically selected and measured, except for teliospores, which required manual elimination of the pedicel and separation of images of adhering spores. Length and width were determined as the major and minor axes of the best fitting ellipse for each spore. This procedure gave values for length and width close to results obtained with an ocular micrometer. Projection area was determined as the number of pixels within spore boundaries multiplied by the area represented by each pixel, giving values that are not feasible to obtain accurately with an ocular micrometer. Of the species studied, spores of *P. recondita* had the largest dimensions, *P. triticina* had the smallest. The rank of the six species based on increasing width, length or projection area was almost the same, using each spore type except pycniospores. Generally, differences of 5% in a given spore dimension between two species were significant. Differences between species were greater with basidiospores and aeciospores than with other spore types. Teliospores were unique in that length and width were negatively correlated, resulting in less variation in area than in length or width. The results indicate that image analysis is useful for measuring spore dimensions, that projection area of spores is a useful added parameter for characterizing rust species and that dimensions of teliospores, basidiospores, aeciospores and urediniospores each are potentially useful for differentiating species.

**Key words:** aeciospore, basidiospore, coronata, graminis, hordei, pycniospore, recondita, striiformis, teliospore, triticina, urediniospore

**INTRODUCTION**

The taxonomy of the Uredinales within genera is based largely on morphological characters, together with range of hosts, including alternate hosts of heteroecious rust fungi. Among useful morphological traits are length and width of teliospores. Rust manuals generally give teliospore dimensions, usually including the range most commonly encountered and the maximum range. Dimensions of other spores in the life cycle also are given sometimes, especially for urediniospores, which often are readily available.

For *Puccinia* species on cereal hosts, spore dimensions and host range both tend to overlap between species, obscuring species boundaries. Distinctive morphological traits such as projections on teliospore caps of *P. coronata* or the extraordinarily long teliospores of *P. eile* are useful when present. For most species, however, the more accurately spore dimensions can be determined the better will be the delineation of species. Spore dimensions have also been used to define groups within species such as subspecies (Savile 1984).

Since their advent in recent years, software programs for digital image analysis have been used to investigate several aspects of fungus morphology and development. These include dimensions and shape of spores (Mitchell et al 1997, Benyon et al 1998), spore numbers (Jones et al 1992), spore germination (Paul et al 1993, Oh et al 1996), germ tube length (Hilber and Schüpp 1992), length and branching of hyphae (O'Shea and Walsh 1996) and size of mycelial aggregates (Adams and Thomas 1988, Cox and Thomas 1992, Tucker et al 1992). Likewise, image
Table I. Number and country of origin of isolates of six species of Puccinia

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin and number of isolates</th>
<th>Total number of isolates</th>
<th>Hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Telial/uredinial</td>
</tr>
<tr>
<td><em>Puccinia coronata</em></td>
<td>Israel 2; USA 2</td>
<td>4</td>
<td>Avena sterilis</td>
</tr>
<tr>
<td><em>P. graminis</em></td>
<td>USA 3</td>
<td>8</td>
<td>Elyrigia repens</td>
</tr>
<tr>
<td>f.sp. secalis</td>
<td>Hungary 1; Germany 1; USA 3</td>
<td></td>
<td>Triticum aestivum</td>
</tr>
<tr>
<td>f.sp. tritici</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. hordei</em></td>
<td>Israel 1; Germany 1; USA 1</td>
<td>3</td>
<td>Hordeum vulgare</td>
</tr>
<tr>
<td><em>P. recondita</em></td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td><em>Aegilops longissima</em> type</td>
<td>Israel 2</td>
<td></td>
<td>Aegilops longissima</td>
</tr>
<tr>
<td><em>Ae. ovata</em> type</td>
<td>Israel 1</td>
<td></td>
<td>Ae. ovata</td>
</tr>
<tr>
<td><em>Ae. variabilis</em> type</td>
<td>Israel 2</td>
<td></td>
<td>Ae. variabilis</td>
</tr>
<tr>
<td><em>Secale montanum</em> type</td>
<td>Israel 1</td>
<td></td>
<td>Secale montanum</td>
</tr>
<tr>
<td><em>T. turgidum var. durum</em> type</td>
<td>Morocco 2</td>
<td></td>
<td>T. turgidum var. durum</td>
</tr>
<tr>
<td><em>P. striiformis</em></td>
<td>Israel 4</td>
<td>4</td>
<td>T. aestivum</td>
</tr>
<tr>
<td><em>P. triticina</em></td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td><em>T. aestivum</em> type</td>
<td>Israel 2; China 1; USA 1</td>
<td></td>
<td>T. aestivum</td>
</tr>
<tr>
<td><em>T. turgidum var. durum</em> type</td>
<td>Israel 1; Ethiopia 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Analysis has been used to determine volume of yeast cells (Makarov et al. 1998), budding of yeasts (O’Shea and Walsh 1996) and shifts from yeast-like to filamentous growth in dimorphic fungi (McCarthy et al. 1998).

For rust fungi, Hernandez et al. (2002) used image analysis to measure length and width of urediniospores and teliospores of *P. hemerocallidis*. Johnson et al. (1999) compared urediniospores of *P. menthae* isolates from spearmint and peppermint, showing that 15 of 18 characteristics, based mainly on dimension and shape, differed between isolates of the two groups. In an investigation of several members of the *P. recondita* complex from wheat, wild wheat and rye, image analysis was used to determine the length, width and projection area of teliospores (Anikster et al. 1997). The results showed that the rust collections fell into two groups differing in teliospore size: teliospores of group I (tentatively termed *P. triticina*) were smaller in both length and width than those in group II (the remaining members of the *P. recondita* complex). The differences in teliospore dimensions were most pronounced when comparison was based on area. Coupled with differences in host range and lack of sexual compatibility, the differences in teliospore dimensions contributed to the conclusion that group I was a species distinct from the rest of the *P. recondita* complex. Likewise, spore dimensions obtained digitally were used to assist in the differentiation of groups within the *P. alliurn* complex (Anikster et al. 2004) and to define a new forma specialis of *Puccinia triticina* from *Aegilops speltoides* (Ben Yehuda et al. 2004).

Here we extend investigation of spore dimensions to include a total of six Puccinia species of cereal rusts (Table I), including *P. triticina* and other members of the *P. recondita* complex as investigated earlier. For each species, isolates from several regions of the world were included to ensure thorough representation. Using image analysis, we measured length, width and projection area of all spore types produced by each species (as shown for *P. recondita* and *P. triticina*, Fig. 1). Our objectives were: (i) to evaluate digital image analysis as an aid to measuring spore dimensions, including use of projection area as a potential descriptor for rust fungus species; (ii) to learn how differences in teliospore dimensions among species relate to dimensions of other spore types in the rust life cycle; (iii) to characterize variation in dimensions in populations of a given spore type within individual species. We found to our surprise that length and width of teliospores are negatively correlated (i.e., variation in length was compensated for by opposing variation in width).

Materials and Methods

The number of isolates for the six rust species investigated are provided (Table I) with country of origin and host spe-
cies. Three to eight isolates were used for each species. Methods for growing and inoculating plants and for harvesting spores were as described by Anikster et al (1997). Spores were prepared for image analysis as follows:

**Teliospores.** Leaves from telia were brought in from the field and dried in paper bags on the laboratory bench for about a week. The bags then were stored in a refrigerator at 4 C for 2 mo to 10 y. For image analysis, teliospores were scraped from 10–15 telial clusters and mounted in 50% aqueous glycerol under a cover slip on a glass microscope slide. At least five fields with 10–20 spores/field were measured for each isolate.

**Basidiospores.** Teliospores (in telia) were preconditioned for germination by incubating them 1–6 wk in distilled water at 4 C (Elam et al 1992). The telia then were placed on a wet filter paper on the lid of a Petri dish so that ba-
sidiospores ejected from basidia produced by the germinating teliospores would fall on cover slips on the bottom of the dish. After a sufficient number of basidiospores were ejected, usually after 20 h incubation at 18 C, the cover slips were inverted on a drop of 1% cotton blue in lactophenol on a microscope slide. The slide was heated briefly on an electric hot plate to 40 C; the basidiospores then were viewed under the microscope. For each isolate, spores were measured in 10 or more fields, each with 10–20 spores. (Basidiospores of Fig. 1 were unstained.)

Pycniospores. To harvest pycniospores, pycnia were touched lightly with a cover slip so that nectar (containing pycniospores) adhered as a droplet. The nectar was allowed to dry 10 min until pycniospores were affixed to the cover slip. A droplet of 1% cotton blue in lactophenol was placed on a microscope slide. The cover slip with pycniospores then was inverted and placed so the spores were immersed in the fixative under the cover slip. The slide was heated as described for basidiospores and examined under the microscope. At least four fields, each with about 200 spores, were measured for each isolate. (Pycniospores shown in Fig. 1 were unstained.)

Aeciospores and urediospores. Freshly harvested aeciospores and urediospores were mounted under cover slips on slides in lactophenol at room temperature. A minimum of 10 microscope fields, each with about 20 spores, was measured for each isolate.

Image analysis.—Spore images were obtained by differential interference contrast (DIC) microscopy with a Zeiss Axioskop equipped with 10× eyepieces. A 40× oil immersion Plan-Neofluar objective lens (N.A.1.30) was used for pycniospores; a 20× Plan-Neofluar lens (N.A. 0.50) was used for all other spore types. For analysis, microscope fields were selected in which illumination was uniform and spores were not crowded (Fig. 2). Images were captured with a LIS 700 CCD black and white video camera (Applitec, Israel). Gain and offset were adjusted usually with the automatic option of the camera. Exposure time was usually 1/500 s. In initial experiments, video images were digitized with a 50 Hz (PAL) Quick Capture frame grabber (Data Translation, Marlboro, Massachusetts) in a Macintosh 8100. Later a video capture frame grabber (All in Wonder, ATI Technologies, Markham, Canada) was used with a NEC (Pentium 4-3 1 Mgh) Packard Bell PC computer. The result was a rectangular array of 640 × 480 pixels with gray levels in increments of 0–255 steps. The width of the rectangle, as viewed on the computer screen (Fig. 2), was equivalent to one-half the diameter of the original microscope field.

Spore images obtained with the Macintosh were analyzed with NIH Image Software (version 1.60) written by Wayne Rasband, National Institute of Health, Maryland. Images obtained with the PC were analyzed with Scion Image version 4.02 (Scion Corp., Frederick, Maryland). To illustrate procedures, close-up views of a portion of the analyzed rectangle are shown (Fig. 3) for each spore type. Initial digital images (Fig. 3A, D, G, J, M) were processed as follows. (i) Background was usually subtracted with “2D rolling ball” algorithms, especially when the background was uneven. (ii) An automatic threshold setting (typically 70–150) was used to produce binary images (Fig. 3B, E, H, K, N). Thresholding sets the minimum gray level required for viewing an image. The threshold is set by automatic analysis of gray levels of the current selection histogram. For basidiospores, germ tubes were excluded because the narrow, low contrast base of the germ tube (Fig. 3D) was below threshold gray level (Fig. 3E). (iii) Spores with areas 10% larger or 10% smaller than the smallest spore (as measured in a preliminary subsample) were eliminated. This removed miscellaneous debris as well as images of two or more spores touching each other (except for teliospores as described below). Spores touching frame boundaries were rejected automatically. (iv) Length and width of spores were measured as the major and minor axes of the best fitting ellipse. Area was computed in pixels and displayed in µm² as the sum of all pixels in an object. Spatial calibration of pixels was done with a stage micrometer. Any white spaces within the boundaries of thresholded spores were included automatically in spore area. Dimensions were determined automatically for all suitable spores in the field, except for teliospores, which were measured individually. A white line was drawn across the base of each teliospore to remove the pedicle before measurements (Fig. 3B). Because teliospores tended to stick together, spores in clumps of 2–3 were separated by drawing a white line between them if areas of overlap were judged insignificant (Fig. 3B). Single-celled teliospores (mesospores) were excluded from measurements. Each prepared teliospore image then was measured by touching the image with the "magic wand" tool (of NIH image) and using the best fitting ellipse procedure as used with other spores. Spores that were included in measured samples were numbered automatically (Fig. 3C, F, I, L, O).

Length, width and area measurements were copied to Excel and are reported as the sample mean and the sample
Fig. 3. Preparation of images for analysis for five spore types of *Puccinia recondita*: teliospores (A, B, C), basidiospores (D, E, F), pycniospores (G, H, I), aeciospores (J, K, L), and urediospores (M, N, O). A, D, G, J, M. Initial digital image. B, E, H, K, N. Binary image after background was subtracted and gray levels below threshold were eliminated using software of NIH Image or Scion. C, F, I, L, O. Spores selected for measurement as numbered. For teliospores only (Fig. 3B), images were modified with white lines to separate pedicels from the main spore body and to separate contiguous spores. Close-up views of only a portion of the rectangular fields used for image analysis, as in Fig. 2. Bar = 20 μm, except 10 μm for G, H and I.

The coefficient of variation (CV), which expresses standard deviation as a percentage of the sample mean. In addition, the square root of the spore area and its CV are provided as a way to compare CVs of length and width to CVs as described in Results. Significance of differences in sample means for species was determined by one-way ANOVA using Tukey's test from SPSSa software. The relation between length and width was characterized by the Pearson correlation coefficient. Photographs of spores (Fig. 1) were taken with a Nikon Coolpix 4500 digital camera.
RESULTS

Differences in size and morphology among the five types of spores investigated are shown (Fig. 1) for P. triticina and P. recondita. For both species, there was an approximate 10-fold difference in both length and width between pycniospores (the smallest of the spore types) and teliospores (the largest) (Fig. 1). Teliospores (Fig. 1A, B) were almost all two-celled, had caps formed by apical wall thickenings and had pedicels that usually were broken off but remained intact in P. graminis. The pedicel of P. triticina had a pigmented, cone-shaped deposit in the remnant of pedicel that remained attached to the teliospore (Fig. 1A). Basidiospores (Fig. 1C, D) were asymmetrical with one side more highly curved than the other. Basidiospores produced germ pores by the time the spores were fixed for observation, as described in Materials and Methods. Pycniospores (Fig. 1E, F) were pear-shaped with thin walls. Aeciospores and urediospores (Fig. 1G, H, I, J) were spheroid, except for urediospores of P. graminis (not shown) which were elongated. Urediospore germ pores (Fig. 1I, J) usually were scattered but were equatorial in P. graminis (not shown). Surfaces of urediospores had spines (not clearly visible at the magnification of Fig. 1I, J).

Evaluation of image analysis protocols.—Because surfaces of aeciospores, teliospores and urediospores are hydrophobic, small air bubbles accumulated around each spore if mounted in water, interfering with measurements. To counter this problem, spores were mounted in glycerin (for teliospores) or lactophenol (for the four other spore types). In preliminary trials comparing water to the mounting media used for each spore type, the media had no detectable effect on spore dimensions.

For all five spore types of an isolate of P. recondita, values for spore length and width obtained by image analysis were compared to values obtained directly with an ocular micrometer on the microscope and also to values obtained by using a ruler on the computer screen before the thresholding step. For measurements with the ocular micrometer, a 100× objective lens was used. For digital images a 20× objective lens was used except 40× for pycniospores, as in our standard protocol. Larger numbers of spores were present for measurement in individual microscope fields at 40× (used for pycniospores) or 20× (used for the other spore types) than at 100×, aiding rapid analysis of spore populations. Results for the three methods agreed within 7% of values obtained with the ocular micrometer (Table II). This indicated that the thresholding step, the use of axes of the best fitting ellipse for length and width and other aspects of the image analysis procedure gave satisfactory results.

Projection area.—Mean projection areas for each spore type in order of increasing values among the species investigated are provided (Table III). The order was almost the same whether based on values for the teliospores, basidiospores, aeciospores or urediospores but not if based on values for pycniospores. Mean values ranged from 6.71 μm² for pycniospores to 719 μm² for teliospores. Among species, areas were largest for P. hordei and P. recondita and smallest for P. triticina for all spore types except pycniospores, which, in contrast, were larger for P. triticina than P. recondita.

Examples of the distribution of area values within spore types are shown in histograms (Fig. 4) for P. triticina and P. recondita, representing the species with the smallest and largest teliospores. Although the spore populations of the two species overlapped, their means were significantly higher for P. recondita than P. triticina for all spore types except pycniospores (Table III). Generally differences in area of a given spore type between any two species were significant if mean values differed by 5% or more.

Length and width.—The rank of species according to increasing length or width usually paralleled rank by
TABLE III. Dimensions and length-width correlation of five spore types of *Puccinia* species as measured by image analysis. Data are for all spores of all isolates for each species (TABLE I) combined into a single population. Species ranked in order of increasing means for area of each spore type.

<table>
<thead>
<tr>
<th>Spore type/species</th>
<th>Number of spores</th>
<th>Area, (\mu\text{m}^2)</th>
<th>(\sqrt{\text{Area}}, \mu\text{m})</th>
<th>Length, (\mu\text{m})</th>
<th>Width, (\mu\text{m})</th>
<th>Length-width correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teliospores</td>
<td></td>
<td>Mean(^a) CV</td>
<td>Mean CV</td>
<td>Mean CV</td>
<td>Mean CV</td>
<td>Mean CV</td>
</tr>
<tr>
<td><em>P. triticiina</em></td>
<td>279</td>
<td>583 a 15</td>
<td>24.1 a 7</td>
<td>45.9 a 17</td>
<td>16.3 a 10</td>
<td>-0.49</td>
</tr>
<tr>
<td><em>P. striiformis</em></td>
<td>110</td>
<td>703 b 15</td>
<td>26.4 b 7</td>
<td>49.4 b 13</td>
<td>18.1 b 10</td>
<td>-0.22</td>
</tr>
<tr>
<td><em>P. coronata</em></td>
<td>78</td>
<td>713 b 13</td>
<td>26.7 b 7</td>
<td>52.1 c 15</td>
<td>17.8 b 14</td>
<td>-0.51</td>
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<tr>
<td><em>P. graminis</em></td>
<td>241</td>
<td>760 c 10</td>
<td>27.5 c 7</td>
<td>49.5 b 13</td>
<td>19.8 c 12</td>
<td>-0.66</td>
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<tr>
<td><em>P. recondita</em></td>
<td>253</td>
<td>771 c 13</td>
<td>27.4 c 7</td>
<td>49.1 b 12</td>
<td>20.1 c 12</td>
<td>-0.37</td>
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<td><em>P. hordei</em></td>
<td>70</td>
<td>784 c 17</td>
<td>27.9 c 9</td>
<td>45.6 a 13</td>
<td>22.1 d 16</td>
<td>-0.34</td>
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<tr>
<td>Mean</td>
<td>719</td>
<td>13.8 26.3 7.0</td>
<td>48.6 13.8 19.0 12.3</td>
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<td>Basidiospores</td>
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<td>Mean CV</td>
<td>Mean CV</td>
<td>Mean CV</td>
<td>Mean CV</td>
</tr>
<tr>
<td><em>P. triticiina</em></td>
<td>730</td>
<td>41.0 a 19</td>
<td>6.37 a 10</td>
<td>8.27 a 11</td>
<td>6.28 a 12</td>
<td>0.30</td>
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<tr>
<td><em>P. graminis</em></td>
<td>564</td>
<td>49.9 b 20</td>
<td>7.03 b 10</td>
<td>9.83 b 15</td>
<td>6.46 b 12</td>
<td>0.04</td>
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<tr>
<td><em>P. coronata</em></td>
<td>261</td>
<td>59.7 c 19</td>
<td>7.70 c 9</td>
<td>10.3 c 12</td>
<td>7.10 c 11</td>
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<td>212</td>
<td>60.1 c 23</td>
<td>7.68 c 13</td>
<td>10.6 d 18</td>
<td>7.40 d 12</td>
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<tr>
<td><em>P. hordei</em></td>
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<td>69.1 d 21</td>
<td>8.27 d 10</td>
<td>12.0 c 13</td>
<td>7.29 d 14</td>
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<tr>
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<td>8.35 d 10</td>
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<td>7.59 c 13</td>
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<td>Mean</td>
<td>59.0</td>
<td>20.2 7.60 10.3</td>
<td>10.5 14.0 7.03 12.3</td>
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<tr>
<td>Pycniospores(^b)</td>
<td></td>
<td>Mean(^a) CV</td>
<td>Mean CV</td>
<td>Mean CV</td>
<td>Mean CV</td>
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<td>1803</td>
<td>7.28 d 30</td>
<td>2.69 e 15</td>
<td>4.12 e 21</td>
<td>2.26 c 15</td>
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<td>25.2 2.57 12.6</td>
<td>3.89 17.0 2.41 15.8</td>
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<td>16.4 a 8</td>
<td>20.1 a 9</td>
<td>17.1 a 10</td>
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<td>299 b 21</td>
<td>17.2 b 10</td>
<td>21.3 b 14</td>
<td>17.9 b 9</td>
<td>0.47</td>
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<tr>
<td><em>P. coronata</em></td>
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<td>312 c 21</td>
<td>17.6 c 10</td>
<td>21.3 b 11</td>
<td>18.6 c 11</td>
<td>0.67</td>
</tr>
<tr>
<td><em>P. recondita</em></td>
<td>693</td>
<td>335 d 19</td>
<td>18.2 d 9</td>
<td>22.1 c 11</td>
<td>19.2 d 11</td>
<td>0.57</td>
</tr>
<tr>
<td><em>P. hordei</em></td>
<td>199</td>
<td>431 e 19</td>
<td>20.7 e 10</td>
<td>25.3 d 11</td>
<td>21.6 e 11</td>
<td>0.56</td>
</tr>
<tr>
<td>Mean</td>
<td>317</td>
<td>19.2 17.7 9.4</td>
<td>21.6 11.2 18.6 10.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urediospores</td>
<td></td>
<td>Mean(^a) CV</td>
<td>Mean CV</td>
<td>Mean CV</td>
<td>Mean CV</td>
<td>Mean CV</td>
</tr>
<tr>
<td><em>P. coronata</em></td>
<td>204</td>
<td>370 a 21</td>
<td>19.1 a 11</td>
<td>23.1 a 12</td>
<td>20.2 b 11</td>
<td>0.75</td>
</tr>
<tr>
<td><em>P. triticiina</em></td>
<td>613</td>
<td>375 ab 17</td>
<td>19.3 ab 9</td>
<td>23.2 a 11</td>
<td>20.4 c 9</td>
<td>0.49</td>
</tr>
<tr>
<td><em>P. striiformis</em></td>
<td>365</td>
<td>380 ab 18</td>
<td>19.4 ab 9</td>
<td>24.4 b 12</td>
<td>19.7 b 10</td>
<td>0.30</td>
</tr>
<tr>
<td><em>P. graminis</em></td>
<td>440</td>
<td>388 b 17</td>
<td>19.6 b 8</td>
<td>28.3 d 14</td>
<td>17.5 a 12</td>
<td>-0.15</td>
</tr>
<tr>
<td><em>P. hordei</em></td>
<td>150</td>
<td>421 c 13</td>
<td>20.5 c 7</td>
<td>24.4 b 14</td>
<td>21.9 d 7</td>
<td>0.61</td>
</tr>
<tr>
<td><em>P. recondita</em></td>
<td>365</td>
<td>489 d 11</td>
<td>22.1 d 6</td>
<td>26.2 c 6</td>
<td>25.7 e 7</td>
<td>0.48</td>
</tr>
<tr>
<td>Mean</td>
<td>404</td>
<td>16.2 20.0 8.3</td>
<td>25.0 11.5 20.6 9.3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Values in a given column followed by the same letter did not differ significantly \((P > 0.05)\).

\(^b\) Pycniospores and aciospores were not available for *P. striiformis*.

measuring area for basidiospores, pycniospores or aciospores, although the position of species with largest spores, *P. hordei* and *P. recondita*, sometimes switched (TABLE III). The ranking with pycniospores, although different from ranking based on other spore types remained almost the same when ranked by length or width as by area. With urediospores, the order by length and width also differed from that by area, mainly because *P. graminis* had relatively long (283 \(\mu\text{m}\)) and narrow (17.5 \(\mu\text{m}\)) urediospores compared to other species. Further the urediospores of *P. graminis* had a low length/width corre-
lation coefficient (-0.15) compared to those of other species (0.30–0.75) (Table III). Increases in length were not associated with increases in width in *P. graminis* as they tended to be in urediniospores of other species.

For similar reasons, the rank of teliospores by increasing length did not relate to ranking by area (Table III). Teliospores of all species had highly negative length/width correlation coefficients. This indicated that teliospore width decreased as length increased. This is illustrated by teliospore length/width plots for *P. graminis* (correlation coefficient, -0.66) and *P. striiformis* (correlation coefficient, -0.25) (Fig. 5).

Therefore rank of species by order of increasing length differed from rank by area. For example, teliospores of *P. hordei* ranked with *P. triticina* as having the shortest teliospores, in contrast to being the largest when ranked by area (Table III).

Another consequence of the negative length/width correlation coefficient for teliospores was lower variation within spore populations for area than for length or width. To demonstrate this we calculated the CV of the square root of area so that, like the CVs for length and width, the unit of measurement was μm (and not μm²). The mean CV for square root of teliospore area (for all species combined) was 7.0, compared to 13.8 and 12.3 for length and width respectively (Table III). Note also that the CV of square root area for all species was less for teliospores (7) than for the other spore types (8.3–12.6), which usually had positive length/width correlation coefficients.

**DISCUSSION**

With the assistance of digital image analysis, we compared length, width and projection area of the five spore types produced by five full cycled Puccinia species: *P. coronata*, *P. graminis*, *P. hordei*, *P. recondita*, and *P. triticina* plus the spores produced by *P. striiformis*, which lacks known pycnial and aecial stages. The results indicate that relative spore size among species tends to be the same for teliospores, basidiospores, aeciospores and urediniospores. For example, species that had large teliospores compared to those of other species also had relatively large basidiospores, aeciospores and urediniospores. The complex genetic and physiological factors controlling spore size within a given species apparently act in common in all four spore types.

The size of pycniospores, on the other hand, did not relate to size of the other four spore types. The pycniospore is highly specialized in that it functions in the environment of pycnial nectar in which it fuses to receptive hyphae (i.e., it does not require protec-

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**Fig. 4.** Histograms of values obtained for cross sectional area for spores of *Puccinia triticina* (open bars) and *P. recondita* (closed bars). A. Teliospores. B. Basidiospores. C. Pycniospores. D. Aeciospores. E. Urediniospores. Open arrow indicates mean value for *P. triticina*; closed arrow indicates mean value for *P. recondita*. 
tion from drying out as do the other spore types, nor
does it produce infection structures required for host
ceil wall penetration or entry through stomates, as in
the case of aeciospores and urediniospores). Because
of the similarity in relative spore size (except for pyc-
niospores), differences between species tended to be
similar whether based on teliospores, basidiospores,
aeciospores or urediniospores. However differences
in size between species were greater with basidio-
spores and aeciospores than with other spore types.
Overall these relationships indicate that dimensions
of basidiospores, aeciospores and urediniospores are
potentially as useful as the more commonly used di-
mensions of teliospores in differentiating between
species.

Digital image analysis aided measurement of spore
length and width in that the spores could be mea-
sured more rapidly than if measured individually in
the traditional way with an ocular micrometer on the
microscope. The exception was measurement of tel-
iospores, which individually required removal of the
pedicel from spore images and, in many cases, separa-
tion of images when teliospores adhered to each
other. For spores with relatively simple round or oval
shapes, image analysis considerably speeds measure-
ments of length and width, which is useful if large
numbers of spores are to be measured. This, in turn,
makes it easy to obtain enough spores for statistical
analysis of results.

The principal advantage of image analysis, howev-
er, was the ability to measure projection area. Area
was determined independently of length and width
from the number of pixels within spore boundaries.
This integrated the entire area of each spore regard-
less of shape. Projection area is potentially the most
important single parameter for characterizing spore
dimensions because it combines aspects of length,
width and shape into a single value.

Furthermore, variation among spores tends to be
less for square root values of projection area than for
length or width, especially for teliospores. Although
area measurements have not been used widely to
characterize spores, image analysis makes area readily
available as a potentially useful parameter, whether
for taxonomic, genetic or physiological purposes.

Length and width of all spore types were deter-
mined as the major and minor axes of the best fitting
ellipse for individual spores. The dimensions ob-
tained in this way were similar to those obtained with
an ocular micrometer (TABLE II). We did not eval-
uate alternative methods for determining length, such
as use of the longest chord within the spore image
or the “fiber” length as measured along the medial
axis of the spore (Johnson et al 1999).

Orientation of the spore in the field of view influ-
ences measured spore length and probably projection
area. This was not a problem with teliospores,
which tended to lie with their longest dimension par-
allel to the microscope slide (FIGS. 1A, B, 3A, B). The
same was true for the elongated urediniospores of P.
graminis (not shown). Orientation also was not a
problem with spores with only small differences be-
tween length and width, including urediniospores of
species other than P. graminis and aeciospores of all
species. On the other hand, some basidiospores and
pycniospores were not parallel to the slide when mea-
sured for image analysis.

Basidiospores, allowed to fall onto slides from ger-
minating teliospores (in preparation for measure-
ment), sometimes landed on one end and became
affixed in a near vertical position, remaining in this position when mounted in lactophenol cotton blue. We could not dislodge such basidiospores without disrupting them. In a similar way some pycniospores were not parallel to the slide surface when mounted in lactophenol cotton blue. Nearly vertical pycniospores were eliminated because they were below the minimum area required to be included in the measured spore population. However, some spores at a low angle to the surface were included, which possibly reduced mean length values slightly. Because this error was probably similar for all species examined, we conclude that values obtained by image analysis for length or area of basidiospores and pycniospores (as in Table III) are valid for comparing species.

The length/width ratio (aspect ratio) can be used to differentiate between species. For example, the length/width ratios for P. graminis urediniospores was 1.62 compared to a mean of 1.14 for the other five species (values derived from data of Table III). The length/width ratio was one of 15 parameters Johnson et al (1999) found to be different between two races of P. menthae. Length/width ratios of urediniospores also have been used to distinguish subspecies. Urediniospores of P. graminis subsp. graminis, thought to have evolved on domesticated cereals, had length/width ratios of 1.8–1.9 whereas urediniospores of P. graminis subsp. graminicola, predominantly a parasite of wild grasses, had ratios of 1.4–1.6 (Savile and Urban 1982, Urban and Marková 1984). Clay models were used by Savile and Urban (1982) to estimate volume of urediniospore protoplasts. The resulting volumes of P. graminis subsp. graminis were 1.5–1.9 times greater than for P. graminis subsp. graminicola. Compared to such estimates of volume, area of urediniospores as determined by image analysis likely will be a more direct and useful way to compare subgroups with species. However length/width correlation coefficients also are useful for characterizing spores. Most spores in our investigation had coefficients that were positive or near zero (Table III). Exceptions were the long urediniospores of P. graminis (correlation coefficient −0.15) and teliospores of all six species examined (correlation coefficient −0.22 to −0.66).

For teliospores the reduced width associated with increasing length results in less variation in area (as expressed by variation in square root of area) than in length or width (Table III). As teliospores are formed in the crowded telium, length and width apparently are coordinated toward maintaining a constant projection area and, probably likewise, spore volume. The teliospore usually germinates in the telium, producing promycelia (basidia) and basidiospores there. It is not disseminated in air as are basidiospores, aciospores and urediniospores. Having less need to maintain an aerodynamic shape, the teliospores can vary in length and width, while nevertheless maintaining uniform spore volume in support of ability to produce promycelia and basidiospores.

The basidiospores newly ejected from germinating teliospores frequently produced germ tubes before they were fixed for image analysis. The tubes fortuitously were eliminated in the threshold process used to generate binary images (Fig. 3D, E). This happened because the base of the germ tube near the spore surface was narrow and had low optical contrast. However germ tubes can be eliminated by a skeletonization (erosion) procedure in which pixels are removed from the surface until germ tubes are eliminated (Hilber and Schüepp 1992). Pixels then are restored over the surface of the image, which results in a full size image of the spore without the germ tube.

In addition to revealing patterns in spore dimensions among spore types and among species, the present investigation serves to emphasize projection area as an important parameter for characterizing rust spores. Useful for all spores in the rust life cycle, projection area is especially valuable for describing teliospores. Dimensions and morphology of teliospores are used widely as descriptors for rust species. Because of the negative length/width correlation as described earlier, variation in area is less than variation in length or width, potentially improving differentiation between species, especially when differences in teliospore dimensions are small.

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LITERATURE CITED


