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Comparing Methods of Ploidy Estimation in Potato (*Solanum*) Species

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Abstract
Ploidy manipulation and the resulting need for rapid ploidy screening can be important in potato research and breeding programs. We tested three predictors of ploidy, particularly seeking the quickest, simplest, and most reliable: Chloroplast number per guard cell (C#), guard cell length (GC), and pollen diameter (P), with a total of seven variations in methods of preparation. Time required for each preparation was assessed, and a panel of inexperienced volunteers compared these methods for accuracy using a standard set of coded samples of known ploidy. The common method of counting C# with iodine stain took longer and was no more accurate than observing C# or GC in tap water. GC from tape impressions of the underside of leaves was reliable and has the advantage of permanent slides for later reference. We recommend GC, whether in water, stained, or as tape impressions. GC is significantly different in diploids and tetraploids, but the distributions do overlap, so experience and care in selecting a representative sample of cells contributes to accuracy. The standard measurement of P after staining with aceto-carmine was faster to prep and just as reliable as epidermal methods for some technicians, even with no previous experience. Pursuit of ultra-simplified methods led us to measure P in tap water. Diameters of pollen in plain water are significantly larger, but only for living pollen, suggesting this method might also be developed into a rapid and reliable way to estimate pollen viability.

Resumen
La manipulación de la ploidía y la necesidad resultante de prueba rápida de ploidía puede ser importante en investigación en papa y en programas de mejoramiento. Probamos tres predictores de ploidía, particularmente buscando el más rápido, simple y confiable: El número de cloroplástos por célula guardia (C#), longitud de la célula guardia (GC) y diámetro del polen (P), con un total de siete variaciones en métodos de preparación. Se evaluó el tiempo requerido para cada preparación, y un grupo de voluntarios inexpertos compararon estos métodos para precisión usando un juego estándar de muestras codificadas de ploidía conocida. El método común de conteo C# con tinción de iodo tomó más tiempo y no fue más preciso que la observación de C# o GC en agua corriente. La GC de impresión en cinta del envés de las hojas fue confiable y tiene la ventaja de preparaciones permanentes para referencia posterior. Recomendamos GC, ya sea en agua, teñida, o como impresiones en cinta. GC es significativamente diferente en diploides y tetraploides, pero la distribución se traslapa, de manera que la experiencia y el cuidado en la selección de muestra representativa de células contribuyen a su precisión. La medida estándar de P después del teñido con aceto-carmín fue más rápida de preparar y justo tan confiable como los métodos epidermales para algunos técnicos, aún sin experiencia previa. La búsqueda de métodos ultrasimplificados nos condujo a medir P en agua corriente. Los diámetros del polen en agua simple son significativamente más grandes, pero solo para polen vivo, sugiriendo que este método debería también desarrollarse de una manera rápida y confiable para estimar la viabilidad del polen.

Introduction

Ploidy determination of many individuals is sometimes needed in potato research and breeding. The most conclusive method is counting chromosomes in the root tips or floral buds, but that requires considerable time and experience, as well as toxic chemicals. Other indirect methods like

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measuring DNA in individual cells with a flow cytometer require specialized preparations and equipment. Deuter (1970) outlined the requirements for mass ploidy estimation methods applicable to a breeding program as: 1) accurate, 2) able to give a verdict for a large number of individuals in a short time, and 3) easily applicable—in other words, not require specially-educated staff. We here investigated the efficiency of several previously-reported methods for potato as well as some of our own simplified modifications with respect to those requirements.

Review of Past Work Deuter (1970) reviews the long history of indirect ploidy estimation techniques for sugar beets, citing papers reporting the usefulness of guard cell size (1942), the number of chloroplasts they contain (1953), and pollen diameter (1939). Johnstone (1939) and Swaminathan (1951) showed that tetraploid potatoes have larger guard cells and pollen than diploids. Sax and Sax (1937) observed that the number of stomata per area indicates ploidy, with tetraploids having more than diploids, but advised that length of guard cells was easier to measure. Singsit and Veilleux (1991) found that there are more chloroplasts per guard cell in potato plants grown in vitro. Similarly, Matteij et al. (1992) were able to accurately differentiate 2x, 4x, 6x and 8x genetically similar individuals of potato fusion regenerants by chloroplast numbers. While most workers report that different ploidies have overlapping ranges of chloroplast numbers, Jacobs and Yoder (1989) reported that there was no overlap between the distributions of genetically similar diploid and tetraploid tomatoes. Qin and Rotino (1995) showed guard cell length and number of chloroplasts accurately estimated ploidy in pepper, as did Sari et al. (1999) in watermelon. Most recently, Ordoñez et al. (2017) recommend chloroplast counting for potato germplasm using an iodine-based stain, and Alsahlany et al. (2019) report that this technique's predictions coincide well with those from single cell DNA measurements by flow cytometry, and with SNP allele dosages (some of which are only possible in tetraploids).

Microscopic anatomy of leaf epidermis has long been studied across many taxa as "peels" of collodion film impressions (Long and Clements 1934), providing an opportunity for another method to assess guard cell size.

Pollen diameter has also been shown to accurately estimate ploidy across a broad range of potato species (Bamberg and Hanneman 1991 and references therein).

Materials and Methods

Seven Methods of Preparations The methods examined were epidermal peels in iodine stain (Ordoñez et al. 2017) and measuring guard cell length (GC-Iodine) and counting the number of chloroplasts (C#-Iodine), epidermal peels with tap water and measuring guard cell length (GC-Water) and number of chloroplasts (C#-Water), tape impressions of undersides of leaves (Kleinhenz et al. 1995) and measuring guard cell lengths (CG-Tape), pollen stained with aceto-carmine (Bamberg and Hanneman 1991) and measuring diameters (P-AC), and pollen treated with tap water and measuring diameters (P-Water).

For GC-Iodine and C#-Iodine, the epidermis of the underside of terminal leaflets was peeled back using a forceps under a dissecting scope, placed on a microscope slide with a drop of stain (one gram each of iodine and potassium iodide dissolved in 100 ml of 70% ethanol), and coverslip applied. For GC-Water and C#-Water, a second set of epidermal peels was made, but tap water was applied in place of the iodine stain. For GC-Tape, the method of Kleinhenz et al. (1995) was used: Applying a piece of Scotch® tape to a slide, then dripping on acetone, shaking off the excess liquid, and pressing the underside of leaves into the softened tape for ten seconds. Slides without coverslips were then placed into a warm air (65C) dehydrator for five minutes to accelerate drying. When the slides were removed from the dehydrator, the leaf was pulled away from the tape. For the two methods based on pollen diameters, pollen was buzzed from fresh flowers directly onto a slide, and liquid and cover slip applied, waiting at least five minutes before observation (Bamberg and Hanneman 1991). Aceto-carmine stain (½% carmine *w/v* dissolved in 45% acetic acid) was used on the pollen for the P-AC method; tap water was used for the P-Water method.

Measurements All measurements were made with reference to units of 1 mm actual length on an ocular micrometer. Chloroplasts were measured at 400x so objects in the field of view were 25 μm per unit, and pollen was measured at 1000x so were 10 μm per unit. C# counts were within individual guard cells.

Approaches to Assessing the Methods Three basic approaches were used: 1) a broad survey of many 2x and 4x germplasm taxa, 2) examination of variations within and among ploidies and techniques, 3) trials of preparation time, scoring time, and accuracy.

Approach 1.– Broad Survey of Estimation Parameters The lead author examined many wild and cultivated potato species of known ploidy growing in the greenhouse and screenhouse at the genebank in the summer of 2017, relying particularly on diploids and tetraploids in a standard "mini-core" set of 25 species representing a spectrum of potato diversity (Hardigan et al. 2015), and also pairs of diploids and their artificial (colchicine-induced) tetraploids available at the genebank. This provided experience and skill in slide preparation and scoring technique and helped set standard ranges

typical for diploids and tetraploids regardless of taxon for each method.

Approach 2.—Variability within Methods within a Single Ploidy About 200 cells were measured from a single plant of tetraploid *S. fendleri* for each of the seven methods, and variability within methods was assessed. A replicated study of distribution of cells of 2x and 4x plants within *S. tuberosum* was also done to compare the distribution curves of cells (for P-AC, GC-Water, and C#-Water), and the frequency of measurements occurring in both ploidies was calculated. Finally, in light of the overlap of 2x and 4x cell distribution curves, coded samples were scored to test the effects of attempting to identify representative cells for measuring instead of measuring random cells.

Approach 3.– Preparation and scoring time, and accuracy Having gained familiarity with preparation and scoring in Approach 1, the lead author prepared 12 slides for each of the 7 methods in three replicated trials on consecutive days. The time to prepare, estimate ploidy, and accuracy was recorded for each method. Next, four genebank employees served as inexperienced volunteers for scoring 10 coded slides, premade by the lead author, for each of the 7 methods. Volunteers were first given labeled 2x and 4x slides as a standard for comparison, and a very basic instruction sheet giving the expected metrics for each ploidy and method (Fig. 1). Volunteers were told only that these sets of ten slides had some diploids and some tetraploids. Volunteers were free to observe as many cells and take as much time as they desired, but told that an accurate verdict was expected after observation of 25 cells or less, and no notes or calculations were recommended as necessary. Statistical significance differences in accuracy of volunteers and methods were determined by simulation in Microsoft Excel® with 10,000 sets of the four random volunteers' observed results, and 10,000 sets of 10 random observed methods' results, respectively.

Findings Unrelated to Ploidy Finally, this work suggested possible association of pollen viability and P-Water, and the association of subtle GC distribution differences within different taxa of a single ploidy. Details of the methods used for preliminary tests on these topics are presented in the following Results and Discussion section.

Results and Discussion

Approach 1.– Broad Survey of Estimation Parameters Figure 1 shows the results of the broad germplasm survey to determine the typical ranges of diploids and tetraploids for the parameters for each ploidy estimation method. There was no notable effect of stain type on perceived C# or CG. In contrast, P-water was substantially larger than P-AC. These standards were used to coach the volunteers in ploidy estimation accuracy trials (Approach 3).

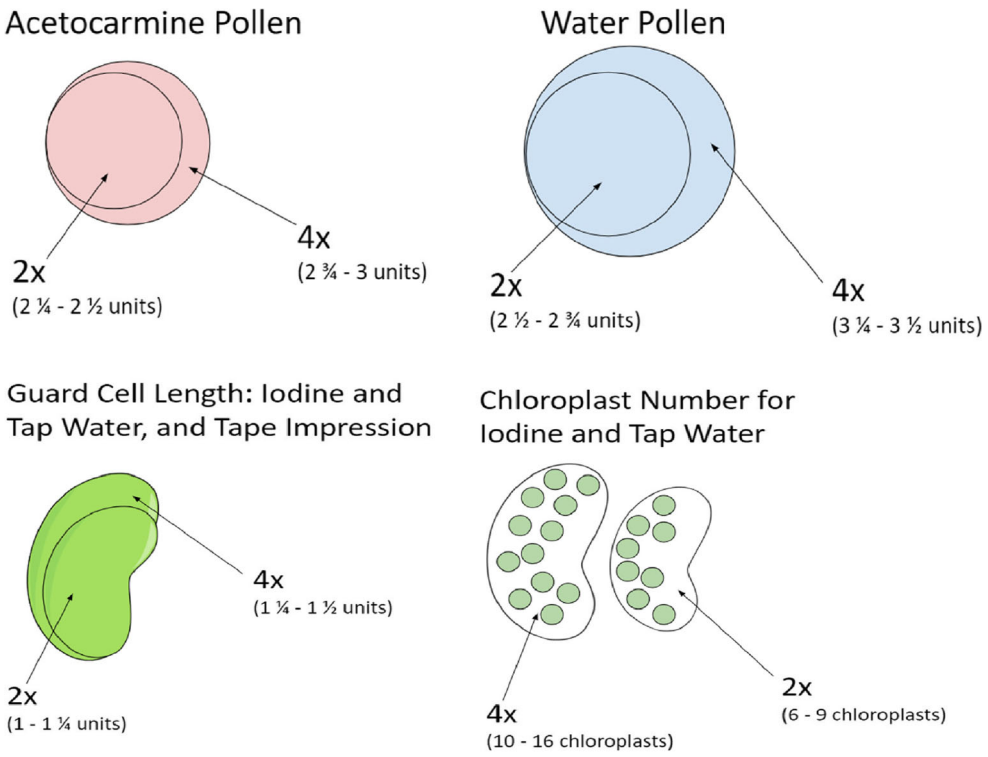

Fig. 1 Typical ploidy estimation parameters derived from a broad survey of diploid and tetraploid germplasm. Units for guard cells are 25 μm, and for pollen are 10 μm

Approach 2.—Variability within Methods within a Single Ploidy The distribution of measurements within a single sample was assessed by examining 200 cells of a single species (4x *Solanum fendleri*) by each method (Figs. 2a-c). Curves were similar in shape for all methods. GC methods also had similar means. In contrast to results of the broad assay of Approach 1, C#-Water mean was a highly significant 1.3 chloroplasts greater than C#-Iodine. As expected from Approach 1 observations, P-Water mean was a highly significant 0.6 units greater than P-AC.

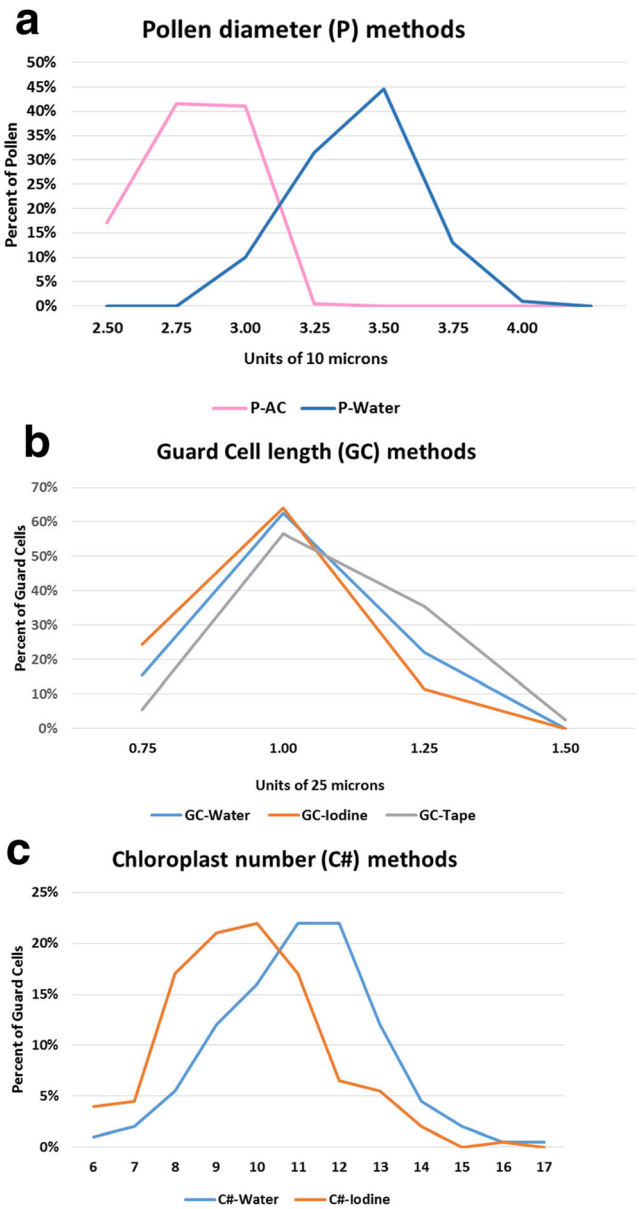

Fig. 2 a-c Comparison of distributions of 200 measurements using the seven ploidy estimation methods using a single plant of *Solanum fendleri*, a tetraploid wild species

Replicated Scoring and Plotting of Paired *S. tuberosum* Samples of Different Ploidies P-AC, GC-Water, and C#-Water distributions of 400 observations per replicate are presented as Figs. 3a-c below. Expected percent ambiguous cells in the 2x vs 4x overlap zones are as expected if equal numbers of random cells of both ploidies were observed. The smallest overlap between 2x and 4x distributions was observed for C#. But we note that chloroplasts greatly varied in the quality of their appearance as distinct countable dots among epidermal peels and among areas within a single peel, while the boundaries of guard cells are almost always very distinct for measuring of their length. Note the apparent counting bias for 4x samples in Fig. 3c, where we suspect that the C# distribution's failure to fit a smooth bell-shaped curve is an artifact. The broad-based screening of Approach 1 concluded that C#-Iodine and C#-Water results were not much different, but, as noted above in Fig. 2c C#-Water appear to have more chloroplasts. This implies that there is actually a reduced ability to detect discreet chloroplasts when Iodine stain is used. A ploidy verdict using C# may not take much more time than other methods if only a few cells are assessed, but counting C# in many cells is much slower than measuring many CG (see also results of Approach 3).

Effect of Scoring Random Cells Versus Ones Selected as Representative In light of the significant overlap of distributions shown in Fig. 3, we conducted preliminary trials to assess the effect of intentionally selecting cells to measure. For pollen, four diploids and four tetraploids were used (a mix of wild and cultivated stocks). About 200 measurements were made for each ploidy for each method, P-Water, P-AC, and GC-Tape. Duplicate coded trials were done with one aimed at measuring cells randomly and the other attempting to first assess the field of countable cells and select only the most representative sizes for measurement. For both P-Water and P-AC, selecting cells resulted in a large increase in the difference of ploidy means (37%) and a large average reduction in the standard deviation (49%). For GC-tape, selecting cells similarly resulted in a large increase in the difference between ploidy means (86%) and a large average reduction in the standard deviation (27%). The implication is that much more experimentation is needed to determine factors important for a representative sample. Of course, to optimize the separation of ploidies, one would theoretically measure many cells and calculate the mean. But a practical microscopic ploidy estimation method needs to rapidly choose and measure a very small proportion of viewable cells, and, ideally, make an accurate judgment without any calculations. The demonstration that ploidy means are separated, and standard deviation lowered by

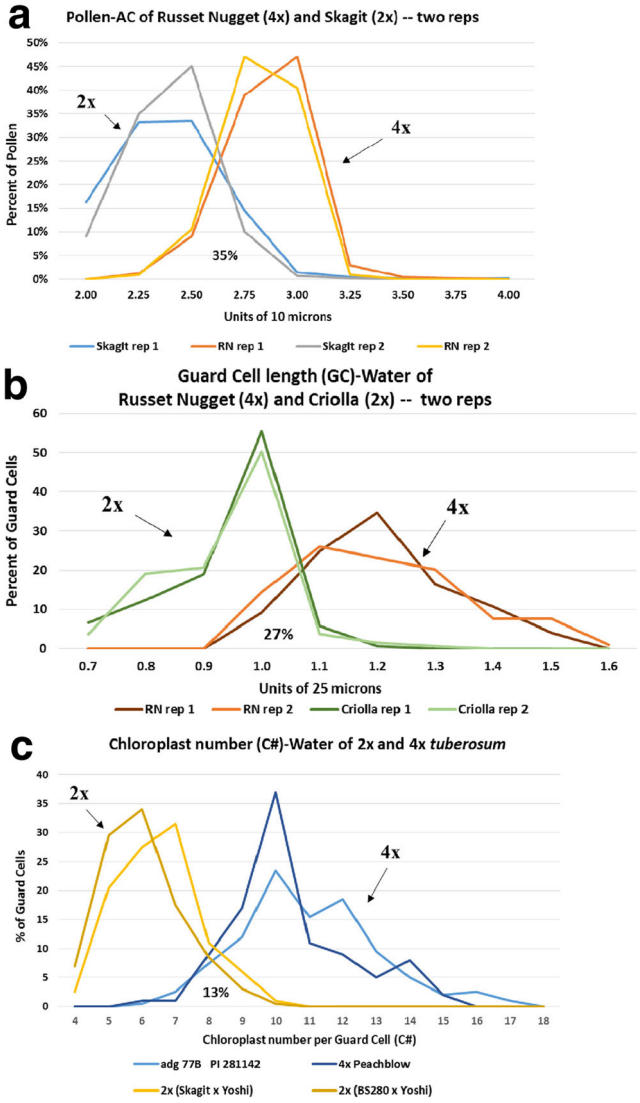

Fig. 3 a-c Distribution curves of P-AC, GC-Water and C#-Water for paired 2x and 4x *tuberosum* samples. Details on materials can be obtained at https://npgsweb.ars-grin.gov/gringlobal/search.aspx with codes RN = AV64, Skagit = BS 296, adg 77B = 281,142, Peachblow = 483,266, BS 280, Yoshi = 654,351. Criolla = complex pedigree breeding line with orange flesh

an experienced technician intentionally choosing certain cells to measure suggests that experience and subjective judgment can make an important contribution to accuracy.

Approach 3.– Preparation and Scoring Time, and Accuracy Trials by Experienced Lead Author The time required to prepare and score 12 slides was compared for the seven methods as three independent trials replicated in three successive days. Samples representing ploidies were not necessarily of the same germplasm each day. ANOVA was used to assess differences. There were no significant differences between days for time of preparation or scoring. Average time of preparation was significantly longer for all epidermal methods (average 3–4 min per slide) versus the pollen methods (average of ~1 min per slide). Tape impression slides were intermediate in time required to prepare (~2.5 min). As noted, pollen size in P-water method was larger and more variable in the initial tests, later suspected to be due to differences in pollen viability. Thus, in these systematic time trials, scoring time and accuracy of P-Water was very variable, so that method was removed from the analysis. Excluding the P-water method, the scoring time required for both C# methods was significantly longer than both GC-Water and GC-Iodine methods (but only an average of about one minute longer per slide– 2.7 min versus 1.7 min, respectively). The accuracy of the lead author's ploidy verdicts using comparable slides is shown on the bottom line of Table 1.

Scoring Accuracy by Inexperienced Volunteers Table 1 summarizes the pattern of significant differences in average accuracy both for volunteers and methods when scoring a standard set of coded, pre-made slides. While fastest to prepare, accuracy with pollen methods was lowest, especially P-Water, for both the lead author and volunteers. This is in contrast to previous reports of pollen diameter accuracy (Bamberg and Hanneman 1991). This might be explained if the particular materials, growth stage, and growing environment have different optima for accuracy when using pollen versus epidermal tissue, another worthy topic for future work. Deuter (1970) showed that chloroplast numbers were less variable in cotyledons than mature leaves of sugar beets and also compared accuracy with different sample sizes, concluding that sample variation approaches a minimum when at least 15 cells are observed.

On the other hand, the significant difference in overall volunteer accuracy particularly was due to some volunteers being able to score pollen with high accuracy. As previously mentioned, a practical estimation method requires rapid measurement of a very small proportion of all the cells available. So the choice of cells considered representative, and the possible subliminal impression and interpretation of the surrounding cells not counted or other such factors introduces a degree of subjectivity. Thus, ploidy estimation may involve unconscious recognition of categories similar to what we described as "cogs" with respect to appearance of the whole potato plant (Bamberg et al. 2016b). Perhaps optimizing that perception is more important for pollen. This might be expected considering that many free-living pollen grains are commonly aborted or otherwise inviable in pollen samples, while one does not expect dead guard cells to be common when embedded in the epidermal matrix peeled from a healthy leaf. Ploidy chimeras in epidermis are possible, but "unreduced" or 2n pollen is actually *common* in some diploid potato germplasm (Spooner et al. 2014) so needs to be ignored for P methods to be accurate. Also, in contrast to epidermal cells, pollen grains might sort by size on the microscope slide, skewing the observed sample. If

Table 1 Accuracy of inexperienced volunteers

	P-Water	P-AC	GC-Tape	GC-Iodine	GC-Water	C#-Iodine	C#-Water	AVE.	p<5%
Volunteer 1	9	9	10	10	10	10	9	**96%**	a
Volunteer 2	8	9	10	8	10	9	10	**91%**	ab
Volunteer 4	7	7	9	9	10	10	9	**87%**	b
Volunteer 3	5	5	7	9	9	9	9	**76%**	c
Average	**73%**	**75%**	**90%**	**90%**	**98%**	**95%**	**93%**		
p<5%	A	A	B	B	B	B	B		
(Author)	*	92%	75%	100%	97%	94%	100%		

Number of correct ploidy determinations of ten samples. Methods and volunteer average accuracy with different letter designations are significantly different at $p < 0.05$. P = pollen, AC = Aceto-carmine, GC = guard cell length, C# = number of chloroplasts per GC. Volunteers are presented in order of accuracy. For comparison, the bottom line gives lead author's accuracy in replicated time and accuracy trials using different materials. Volunteers and methods designated with different letters are significantly different

*not recorded due to very high variability (caused by dead pollen).

so, a viscous additive like glycerin could be a simple countermeasure. For pollen, one must wait for the plant to develop flowers, and will not work at all for plants that are completely male sterile. More work is needed to identify the speed of scoring that provides the optimal balance between promptly making a verdict and accuracy for the average person within a given experience level. There was no obvious trend in wrong ploidy verdicts by volunteers, with about 60% of errors being on diploids, and 40% on tetraploids (data not shown). One might suspect that the accuracy of ploidy verdicts when using epidermal methods could be easily improved by adding GC measurements to C# counts for each cell in a single prep—but we did not investigate that possibility or the correlation between the two methods applied to the same cells. Deuter (1970) showed that in sugar beets, accuracy could be substantially improved by applying multiple methods. It could be interesting to find out if people count significantly more C# in photographs of the same guard cells made to look artificially bigger, even without an obvious standard dimension in the field of view.

Findings Unrelated to Ploidy– P-Water as a Test of Pollen Viability AC staining has long been used to estimate pollen viability, but is not a vital stain per se, only an indication that pollen did not abort before full development. In the initial broad surveys of Approach 1, P-Water ploidy estimates were made on many pollen samples, including some that had been

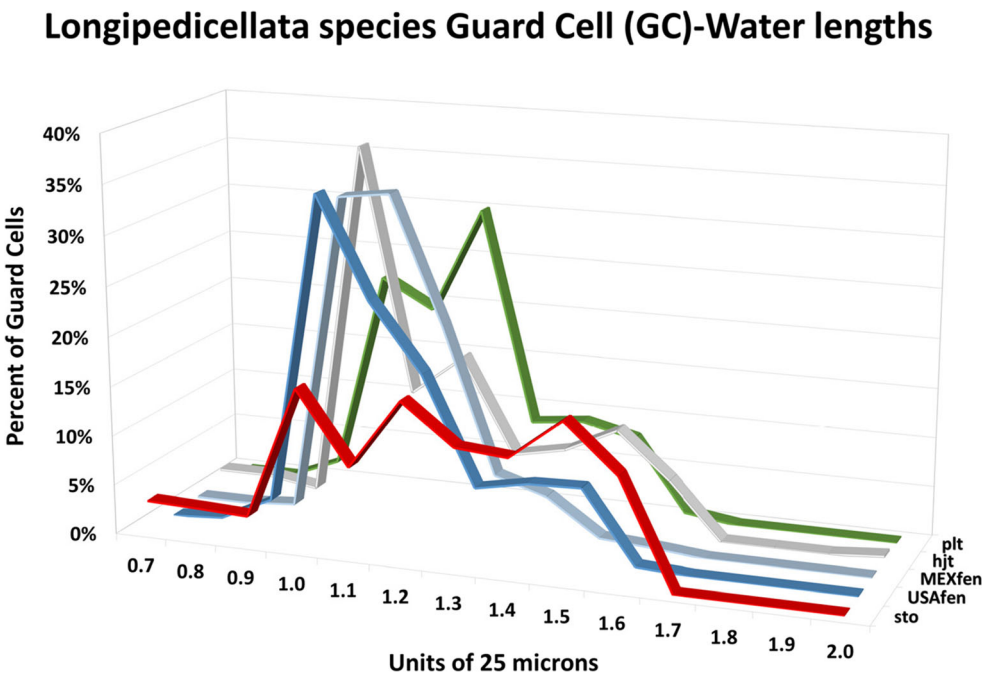

Fig. 4 Distribution of mean GC in subgroups of Series Longipedicellata. *Solanum* species sto = *stoloniferum*, fen = *fendleri*, hjt = *hjertingii*, plt = *polytrichon* (all tetraploids)

held for almost one year in the refrigerator. These were remnants of samples collected for unrelated projects and had not been discarded despite being assumed to be too old to be viable for successful pollinations. Implication of pollen viability as a factor came to light when P-Water diameters of tetraploids were much smaller than expected, often resulting in them being declared to be diploids. We investigated this phenomenon further by measuring pollen of four diploid wild species and one cultivated tetraploid assumed to have high viability due to robust pollen shed and high percent stainability with AC (data not shown). Identical pollen samples were either fresh (assumed viable) or heated for four hours in an oven at 65C (assumed dead). When dead, pollen size in P-Water was always exactly the same as in P-AC. In contrast, fresh pollen diameter was always significantly greater in P-Water than P-AC, with diameter swelling averaging 24%. Thus, in ten separate observations, pollen swell only associated with the five presumed live pollen samples, a result expected to occur by chance at $p < 0.001$. These observations suggest that pollen killed by age, heat, or the acetic acid in AC does not swell as does live pollen in tap water. Pollen viability assays can be complicated and inconsistent (Trognitz 1991; Rodriguez-Riano and Dafni 2000). Thus, there may be value in further investigating P-Water as a quick and simple viability assay. Note that the effect of using water can be greater than ploidy: In the *S. fendleri* trial the difference of P-water was 3.81–3.41 = 0.60 units, while the effect of ploidy in the 4x and 2x *tuberosum* trial was 2.84–2.39 = 0.45 units. There is much literature on pollen swelling as an indicator of potential for growth and fertilization, but we detected no previous reports using the *degree* of swelling to estimate viability.

Findings Unrelated to Ploidy– Subtle Differences in Taxa of the Same Ploidy A rapid screen of the ploidy of many individuals cannot require the measuring of many cells per slide. But measuring many cells for a thorough assessment of the size distribution might have other applications like distinguishing different taxa of the same ploidy. A separate study aimed at characterizing subgroups within tetraploid Series Longipedicellata was in progress (Bamberg et al. 2016a), providing ideal uniformly-grown leaf tissue for GC measurement. Thus, we examined tissue of one seedling from each of four populations from each of five Longipedicellata subgroups (*S. fendleri* from the USA, *S. fendleri* from Mexico, *S. polytrichon, S. stoloniferum, S. hjertingii*). At least 60 GC measurements were made on each of these 20 individuals, and the frequency of each GC class was calculated. The distribution curve for *S. stoloniferum* looks different from the others (Fig. 4). ANOVA confirmed that the interaction of subgroups with proportion of observations in a given GC size class was highly significant. When *S. stoloniferum* is removed from the analysis, there is no such significant interaction for the remaining subgroups. This preliminary study suggests GC distributions may have utility as a rapid, low-tech way to evaluate subtle differences among potato taxa.

Conclusions

Guard cell length, actual or from tape impressions, is just as accurate as chloroplast numbers and is faster to score. Ploidy estimation accuracy is just as high with tap water as stains. Pollen comes from sporogeneous tissue and is fastest to prep and score, but pollen methods did not have the consistent high accuracy reported in previous publications. Accuracy of inexperienced volunteers varied significantly. Since distributions of measurements of all random cells of diploids and tetraploids overlap, but intentional selection improves ploidy resolution, the technician's experience and subjective judgment is likely an important component of accuracy, especially for pollen, this being an intriguing topic for future study.

Acknowledgments and Perspectives Thanks to USPG staff Mrs. Renee Sauer, ploidy-scoring volunteers, and the University of Wisconsin Peninsula Agricultural Research Station (PARS) for their help. We observe that even a simple study like this reveals several related facets which deserve more focused attention– for example, the question of how subliminal perception affects the ploidy verdict when the researcher selects and measures only a few cells among many thousands in the field of view. Unrelated ideas, sometimes even more interesting that the original theme may be suggested, like using pollen swelling as an indicator of viability or guard cell size distributions to separate taxa. Isaac Asimov remarked that scientific discovery springs more from a "that's funny" moment than "Eureka!" Similarly, the late legendary UW potato germplasm researcher and former genebank director Stan Peloquin remarked that while we pursue carefully planned-out hypotheses, just being busy working with germplasm and noticing unexpected phenomena that appear is often actually the start of the path to important scientific discoveries.

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