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Dedicated to Prof. dr. sc. ZVONIMIR DEVIDÉ on the occasion of his 80th birthday

Feulgen densitometry: some observations relevant to best practice in quantitative nuclear DNA content determination

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Despite of a huge amount of literature on the Feulgen reaction for DNA relatively few investigations deal with the special needs of plant scientists. As a consequence various modifications of the method are practised in different laboratories, which may in part be responsible for contradictory results. In the present work tests are conducted to find out, which steps of the procedure must be stringently controlled and in which steps such a control is less critical. Test material were mainly root tip meristems of *Pisum sativum*, which were hydrolysed and further processed *in toto*. Some tests involved also *Zea mays*, *Glycine max*, and *Hordeum vulgare*. Fixations may be stored at –20 °C in ethanol for at least 5 years without loss of quality. Nothing is known about permitted conditions of storing fixations at temperatures higher than –20 °C, except that at room temperature a security limit for acetic alcohol fixed material is about 3 days. Staining intensity after fixation with formaldehyde is dependent on concentration, time, and temperature of the fixative. Therefore, co-fixation of test and standard material, if to be included, is strongly recommended, while with acetic alcoholic fixation this point is less critical. Hydrolysis is the most critical step of the procedure. Precise control of HCl molarity, temperature, and optimum time is essential. This is often given not due attention in contemporary publications on plant DNA amounts and Feulgen staining. Hydrolysis curves for methanol acetic acid fixed material with 5 M HCl at 20 °C, 10 °C, and 0 °C show staining optima after 60 min, 220 min, and 20 h, and a plateau of optimum staining about 5 h long in the latter. Hydrolysis optimum (5 M HCl) for formaldehyde fixed material at 20 °C is at 90 min. Washing time after hydrolysis is a surprisingly sensitive step of the procedure and should be kept as short as possible. Time of staining and of all further steps of the procedure, if conducted longer than necessary, lead to a gradual decrease of nuclear dye content. It is suggested to keep these steps as short as possible, because the gradual decay of staining intensity is assumed to proceed not in a stoichiometric fashion. Relatively insensitive steps of the procedure are a final wash in ethanol before drying the slides and storing the slides in the dark.

Key words: Feulgen reaction, hydrolysis, fixation, formaldehyde, DNA, image analysis, densitometry

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Introduction

The Feulgen reaction for DNA (FEULGEN and ROSSENBECK 1924) is one of the most specific cytochemical reactions and therefore still is of great importance to the determination of the DNA content of cell nuclei in cytological preparations. The principle of this type of DNA dyeing consists in a reaction of leucofuchsinic acid (Schiff's reagent) with the reactive aldehyde groups of hydrolytically depurinized deoxyribose, by which the reagent turns purple. There is an enormous amount of literature on technical aspects and factors influencing the results of this quantitative staining procedure. We point in first instance to KRUG (1980), whose book is especially useful to readers capable of the German language. Nevertheless, as sometimes is regretfully noted (HESEMANN 1982), relatively little research has been done concerning the specific needs in the work with plant material. An important contribution is by FOX (1969). BENNETT and SMITH (1976) provided a lucid discussion of potential errors in plant cytophotometry and emphasised the need of including an internal biological standard in the experimental procedure, when interspecific comparisons of DNA amounts are conducted. Virtually every step of the procedure has been investigated from various viewpoints since FEULGEN and ROSSENBECK (1924) mainly in material of medical interest (KASTEN 1960, KRUG 1980), but in plant science even today there are points under discussion, such as the proper choice of the fixative (GREILHUBER 1997) or the required stringency of hydrolytic conditions (GREILHUBER and BARANYI 1999), or the permitted conditions of storing fixations (no specific publication known). Little is known in the literature about the effects of the various steps of the Feulgen procedure, in its various modifications usual in the different laboratories, on the precision and reliability of the measurement results.

The present contribution is intended to provide experimental data that could help in deciding which steps of the procedure must be stringently controlled and in which steps such a control may be somewhat relaxed without loss of quality of the results. We investigated the influence, on Feulgen staining intensity of plant nuclei, of time and temperature of fixing with formaldehyde, of time and temperature of storing fixed material, the time and temperature of washing steps before and after hydrolysis and after the Feulgen reaction, respectively, the staining time and temperature itself, the time in acetic acid before squashing, the influence of a rinse in ethanol for various times after squashing, and the influence of the storing time of stained slides. We present hydrolysis curves for 5 M HCl at 20 °, 10 °, and 0 °C. Some other recommendations are made for best practice. We assume that the observations presented here are useful especially to those who still do not have extensive personal experience with quantitative DNA cytophotometry.

Material and Methods

Most tests were conducted on primary and secondary root tip meristems of *Pisum sativum* cv. Kleine Rheinländerin. *Hordeum vulgare* cv. Ditta, *Zea mays* line CE 777, and *Glycine max* cv. Ceresia were also used in some tests. The seeds were germinated on plates.

The fixative was freshly prepared methanol acetic acid (3:1) or 4% phosphate-buffered (Sörensen pH 7) formaldehyde, which was either freshly prepared as described by HELANDER (2000) from paraformaldehyde, or was used as a working solution that had been

prepared from a commercial stock solution of formaldehyde, as indicated in the Results. Schiff's reagent was prepared from basic fuchsin (Fluka) as described in GREILHUBER and EBERT (1994) according to a formula that is widely used in Great Britain and once was personally communicated to us by R. K. J. NARAYAN (Aberysthwyth). Hydrolysis with adjusted 5M HCl (Merck, Titrisol) was conducted using an ultra-thermostated water bath (for 20 °C and 10 °C) or ice-water in a box filled with ice-chips (for 0 °C). For removing non-bound Schiff's reagent after staining, SO_2 water was made dissolving 0.5 g $K_2S_2O_5$ in 95 ml distilled water and adding 5 ml 1M HCl.

The whole preparative procedure is conducted on root tips *in toto* and consists of (1) fixation, (2) washes to remove fixative, (3) storing, (4) hydration, (5) hydrolysis, (6) washes to remove HCl, (7) Feulgen reaction in Schiff's reagent, (8) washes in SO_2 water to remove unbound reagent, (9) softening in 45% acetic acid, (10) squashing onto glass slides, (11) freezing and removing cover slips, (12) rinsing in 96% ethanol, (13) drying and storing the dry slides. The variations applied to these steps are described in the Results. Hydrolysis curves were established by adding marked root tips to 5M HCl according to a time schedule, so that hydrolysis was stopped for all meristems at the same time and staining occurred simultaneously. Long curves were done as two partial curves, that were linked together by overlapping parts.

The method referred to as »standard Feulgen procedure« consists of a short hydration of fixed/stored *Pisum sativum* root tip meristems, 5M HCl for 60 min at 20 °C, several short rinses (about 10 min), Schiff´s reagent in the refrigerator overnight (12–15 h), 45 min SO_2 water, a short rinse in distilled water and about 10 min 45% acetic acid before squashing, freezing, passing through ethanol, drying, and measurement usually on the same or the next day. If not stated otherwise, per data point in the Figures, 2 slides (10 telo- and 10 prophase nuclei per root tip and slide) were measured.

DNA content determinations were done on the CIRES (Cell Image Retrieval and Evaluation System, Kontron, Munich), release 3.1, a DNA image analysis system primarily designed for studies in human cancer cytogenetics (DIMITROVA et al. 1999, VILHAR et al. 2001). The microscope was a Zeiss Axioskop with a stabilised light source. A neutral density filter and a green interference filter were taken. Measurements were done under a 63 × 1.25 oil iris immersion objective without mounting a cover slip onto the slides. The green channel of the camera (Sony 3CCD colour camera, model DXC-930P with adaptor CMA-D2) was used to grab images (frame grabber KONTRON Smart DFC 500). The CIRES software package was used to process the images with local background determination, i.e., the nucleus was segmented and the light intensity of the reference background was automatically determined from a narrow blank zone surrounding the nucleus. The images were corrected for uneven illumination of the field of view (shading correction). Integrated optical density (IOD) was measured for ten early prophase and ten late telophase nuclei per slide and root tip. A comparison of the performance of the CIRES with other methods of DNA content measurement is given in VILHAR et. al (2001).

ANOVA and correlation analysis were done with SPSS (release 9.0) and Excel 2000. In a few cases lacking points of hydrolysis curves were inserted using the Spline Interpolation DOS program written by Mag. Wilhelm Temsch, Vienna.

Results

In the following the steps of the procedure are analysed with reference to the staining intensity as a performance criterion. Therefore, it is necessary to discuss every point by cross referencing to other points of the procedure.

Fixation and storing fixed material

There are basically only two types of fixative routinely used in Feulgen densitometry, acetic alcoholic fixatives and buffered and dilute formaldehyde. It should be noted in advance, that also formaldehyde fixation results in perfectly spreadable preparations. We studied first the behaviour of methanol acetic acid (MAA) fixed meristems of pea under prolonged storage at –20 °C or at room temperature (21 – 23 °C) (Tab. 1). Fixation was for about 24 h at about 10 °C in the refrigerator. The material was then transferred to 96% ethanol and stored at –20 °C, or kept at room temperature for some time. In one test also non-fixed fresh material was included. The standard Feulgen reaction gave very similar results in material either non-fixed or fixed for up to 5 years when stored at –20 °C (Tab. 1). This demonstrates that (i) hydrolytic behaviour is very similar in non-fixed and MAA-fixed material, and (ii) material may be stored for 5 years at least in ethanol without risk at –20 °C. On the other hand, MAA-fixed material stored for 26 weeks at room temperature gives only much reduced (60% or less) and more variable nuclear staining and shows considerable plasmatic background, and thus is completely useless for quantitative purposes (Tab.1). By extrapolation we would conclude that 3 days at room temperature are a risk limit, which should of course be avoided. In a similar way, previous observations in *Allium cepa* have indicated that the first decay of stainability is seen after one week storage at room temperature (not shown). These data also would indicate, that storing in the refrigerator cannot be prolonged indefinitely, but this has not been evaluated yet. Also still not evaluated is the staining behaviour of the material when kept in the fixative instead of ethanol. We prefer 96% ethanol to reduce the amount of protons and water, both considered to be essential for hydrolysis, which should be avoided.

It is well known that formaldehyde is to be preferred or even necessary as fixative when condensed tannins or other similar secondary compounds are present (GREILHUBER 1986).

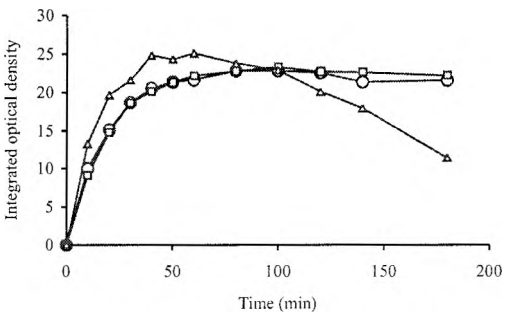

Fig. 1. Hydrolysis curves of *Pisum sativum* root tip meristems, fixed either in MAA (triangles) or in 4% buffered formaldehyde for 1.5 h, stored at –20 °C overnight (squares) or for 69 weeks (circles). The root tips were added to 5 M HCl at 20 °C step by step until after 180 min the hydrolysis was stopped and the root tips were stained jointly.

Tab. 1. Influence of the age of the fixation and storage temperature on nuclear Feulgen staining intensity, studied in primary root tip meristems of *Pisum sativum*. The fixative was MAA (3:1), the storage medium was 96% ethanol. Three tests were conducted, within each of which the data are comparable and are presented normalised, taking the mean of the least aged fixations (†) as 1.000. Per sample 5 slides, 10 telophase and 10 prophase nuclei each, were measured. CV_{in} is the coefficient of variation within slides. Samples marked by * and *** differ significantly at $p = 0.012$ and $p < 0.001$, respectively, from each other, according to hierarchical ANOVA and the Scheffé test.

	Telophases			Prophases		
Storage time and condition	Mean	SD (n=5)	CV_{in}	Mean	SD (n=5)	CV_{in}
Test 1						
Fresh material, unfixed*	1.006	0.015	4.28	1.012	0.014	2.87
† 14 weeks, –20 °C	1.021	0.016	2.39	0.979	0.009	1.84
277 weeks, –20 °C*	1.001	0.017	2.20	0.963	0.016	2.11
Test 2						
278 weeks, –20 °C	1.013	0.028	3.33	1.002	0.036	2.67
149 weeks, –20 °C	1.004	0.028	2.14	0.980	0.167	2.18
† 1 week, –20 °C	1.011	0.019	2.57	0.989	0.007	2.03
Test 3						
† 26 weeks, –20 °C***	1.007	0.024	2.72	0.993	0.016	2.29
26 weeks, room temperature***	0.549	0.041	6.42	0.607	0.027	3.08

Hydrolysis curves shown above (Fig. 1) indicate that with formaldehyde-fixed material the long plateau of staining with an optimum time at 90 min (20 °C) is certainly experimentally advantageous. In comparison, the optimum time is with MAA fixed material at 60 min, the »plateau« is comparatively short, and the maximum staining is stronger.

We applied 4% formaldehyde at several temperatures and for several periods of time (Fig.2). It becomes clear that increased temperature and prolonged time lead to reduced

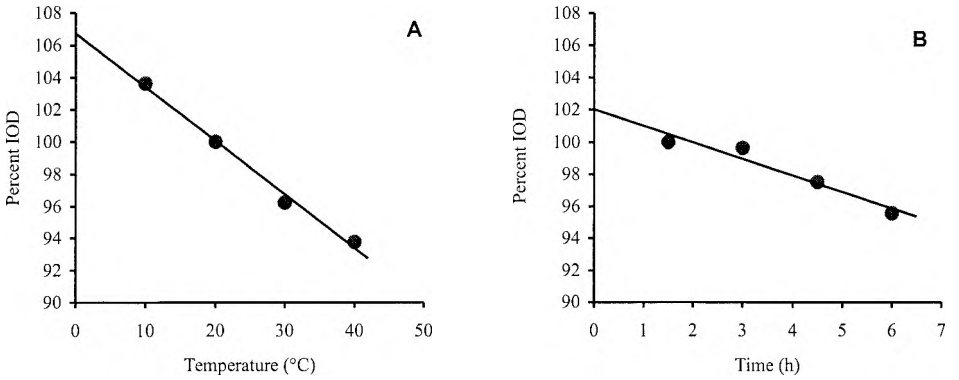

Fig. 2. Influence of temperature (A) and time (B) of fixing root tip meristems of *Pisum sativum* with 4% buffered formaldehyde on the intensity of Feulgen staining. The experiment on temperature included material fixed for 1.5 h, the experiment on time was conducted at 20 °C. The data are normalised taking as 100% stain intensity the value at 20 °C in A, and at 1.5 h fixation time in B. Regressions for A conform to $y = -0.3331x + 106.75$, $r^2 = 0.9918$, and for B to $y = -1.0243x + 102.03$, $r^2 = 0.9363$. IOD – integrated optical density

Feulgen staining, either because aldehyde groups are less accessible or fewer of them are formed. This type of fixation is temperature and time-dependent (and also concentration-dependent, not shown here). From the regression line (Fig. 2) we estimate, that a difference of 5 °C in fixation temperature (say, between 20 °C and 25 °C) would change stainability by about 1.7%. It is thus strongly recommended to co-fix an internal standard, if genome size determinations are to be made, that require the inclusion of a reference material of known DNA amount. However, in another test, pea root tips fixed with freshly prepared formaldehyde or fixed with an old working solution (both 4% phosphate buffered, applied for 1.5 h at 20 °C) behaved very similar in regard of staining intensity (Tab. 2). With reference to a report by HELANDER (1994), that formaldehyde fixation is a reversible process in that it is again removed from fixed tissue upon extensive rinsing in water, a 69 weeks old fixation of pea stored at –20 °C and a recent fixation were compared by hydrolysis curve, and no essential difference was found (Fig.1). This would mean that no dramatic differences in behaviour are to be expected to occur while formaldehyde-fixed material is

Tab. 2. Genome size comparison of *Hordeum vulgare* cv. Ditta and *Pisum sativum* cv. Kleine Rheinländerin by Feulgen DNA image cytometry, using a 4% freshly prepared formaldehyde (FA) solution (»Helander«) and an old 4% FA working solution as fixatives. Fixation was for 1.5 h at room temperature done jointly for *H. vulgare* and *P. sativum*. Hydrolysis and further processing was done in the same test tube for all root tips. The data are normalised taking the grand mean of *Pisum sativum* as 1.000. Per slide and root tip meristem 10 telophases (T) and 10 prophases (P) nuclei were measured. Six slides per species and fixative were measured by the first operator and 5 by the second one. The standard deviation (SD) refers to the means of 11 slides.

	Telophases × 2		Prophases		(2T+P)/2
	Mean	SD	Mean	SD	Mean
Hordeum vulgare					
FA »Helander«	1.043	0.022	1.061	0.021	1.052
Old FA working solution	1.067	0.021	1.077	0.026	1.072
Mean	1.055		1.069		1.062
Pisum sativum					
FA »Helander«	1.003	0.014	1.007	0.017	1.005
Old FA working solution	0.992	0.026	0.998	0.020	0.995
Mean	0.998		1.003		1.000
Ratio *H. vulgare* / *P. sativum*					
FA »Helander«	1.040		1.054		1.047
Old FA working solution	1.076		1.079		1.077
Mean	1.058		1.066		1.062

Hierarchical ANOVA

Variation	DF	F ratio	Probability
Between fixatives	1	0.1661	n.s.
Between operators	2	0.1705	n.s.
Between species	4	33.3395	< 0.001
Between meristems	36	3.9742	< 0.001
Between T × 2 and P	44	5.0823	< 0.001
Between nuclei	792		

stored at –20 °C in ethanol, if formaldehyde concentration, time, and temperature of fixation have been very similar, as in the present case (4% formaldehyde, pH 7, 1.5 h at room temperature).

We compared C-values of *Hordeum vulgare* cv. Ditta and *Pisum sativum* cv. Kleine Rheinländerin in two tests involving 4% formaldehyde as fixative. A joint hydrolysis curve (20 °C) of co-fixed root tips (4% working solution) yielded a very similar run of the curves in the two species (not shown). From the plateau (80 min to 130 min) a DNA content ratio of *H. vulgare versus P. sativum* of 1.088-fold was deduced. I another test (Tab. 2) the two authors measured both species that had been fixed at the same time either with a freshly prepared 4% formaldehyde solution (HELANDER 2000) or a several month-old solution prepared from a commercial solution. Neither authors nor fixations produced different results upon nested ANOVA, but meristems and mitotic phases (calculated of same C-level) differed significantly ($p < 0.001$) and species differed strongly 1.062-fold. *Pisum sativum* and *H. vulgare* behaved in opposite directions with the formaldehyde solutions, indicating that the differences observed occurred by chance. The values for *H. vulgare* are within the range found previously by DOLEŽEL et al. (1998) and VILHAR et al. (2001) with different methods of measurement.

Wash after fixation

Formaldehyde must be carefully removed from the material before storing. Several washes in MAA (for about 1 h), eventually preceded by a short wash in water, are to be preferred over extensive rinsing in water. While in pea root tips and other materials some plasmatic staining remained in cortical layers even after extended washes in water (GREILHUBER and EBERT 1994), such a background completely disappears after this MAA »post-fixation« (suggested first by R. K. J. NARAYAN, pers. communic.).

MAA-fixed material does not need specific washes before being transferred to 96% ethanol for storage.

Preliminary observations indicate, that fixed material is fairly insensitive against prolonged washes in distilled water (two days in the refrigerator should have no adverse effect).

Hydrolysis

It is essential to hydrolyse the material with HCl of controlled molarity at precisely controlled temperature for an optimum time (GREILHUBER and BARANYI 1999). It is also well known, that hydrolysis with 5 M HCl at low temperatures is superior to the classical hydrolysis with 1M HCl at 60 °C, because the latter shows an exceedingly small time frame in which optimum staining occurs (ITIKAWA and OGURA 1954, FOX 1969). The use of room temperature without stringent control, such as done by many previous workers (ITIKAWA and OGURA 1954, DEITCH et al. 1968) is not suitable for quantitative purposes (GREILHUBER and BARANYI 1999).

We made 5 M HCl hydrolysis curves with MAA-fixed material at three temperatures, 20 °C, 10 °C, and 0 °C (Fig. 3). In accordance with previous observations (FOX 1969, KRUG 1980, GREILHUBER 1986) the optimum at 20 °C was at 60 min. At 10 °C the optimum was at 220 min and we see, that between 210 and 260 min very little changes in staining intensity

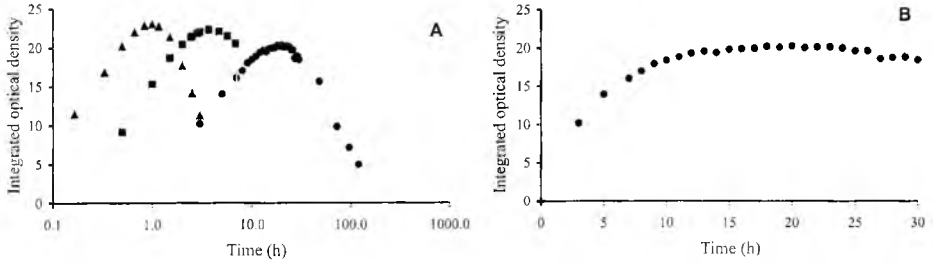

Fig. 3. Hydrolysis curves (5 M HCl) of *Pisum sativum* root tip meristems conducted at 20 °C (triangles), 10 °C (squares), and 0 °C (circles) (A). The first part of the 0 °C curve is shown at a linear time scale in B. Each data point is the mean value of two slides (meristems) of which 10 telophases and 10 prophases were measured. Different Feulgen batches were used for each hydrolysis curve and absolute integrated optical densities (IOD) should not meaningfully be compared.

occur. At 0 °C the optimum was at 20 h with a plateau from 18 to 23 h, in which no essential change in staining intensity is noted. In logarithmic presentation (Fig. 3) these three hydrolysis curves are similar in shape and appear more or less parallel-shifted.

We compared also hydrolysis curves of formaldehyde-fixed material (fresh and stored) with MAA-fixed material (Fig. 1). Formaldehyde-fixed nuclei show somewhat weaker staining than MAA-fixed nuclei, but formaldehyde-fixed material shows very little decay in staining intensity up to 3 h of hydrolysis (5 M HCl at 20 °C). This applies for both, freshly formaldehyde-fixed and stored material, so that the observation by HELANDER (1994), that formaldehyde is a reversible process, apparently does not alter the hydrolytic behaviour of chromatin very much. The optimum time is 90 min for formaldehyde fixation compared to 60 min for MAA fixation.

Wash after hydrolysis

It is necessary to stop hydrolysis by removing HCl, which we used to do by a wash in distilled water at room temperature. We tested the effect of such a prolonged wash up to 4 h after a 20 °C hydrolysis in 5 M HCl after both types of fixation. With MAA-fixed material, there is a drastic decay of stainability between 1 and 2 h (Fig. 4), which is accompanied by a structural deterioration of chromosome integrity and emergence of a pink plasmatic background, which is probably caused by mobilised DNA fragments. The coefficient of variation (CV) within slides becomes larger and the prophase/telophase ratio worse. After 4 h forty percent of dye content is lost. By regression analysis one may estimate 2–3 percent of dye content is lost within 15 minutes.

With formaldehyde fixation the loss of staining is less drastic, but still amounts to about 16 percent within four hours. It is therefore clear, that the washing time after hydrolysis is a critical point of the whole quantitative procedure.

Staining with Schiff's reagent

We investigated the influence of staining time and temperature on the intensity of staining. We stained *Pisum sativum* meristems at 20 °C for 1 to 34 h (1 – 11 h in 1 h intervals)

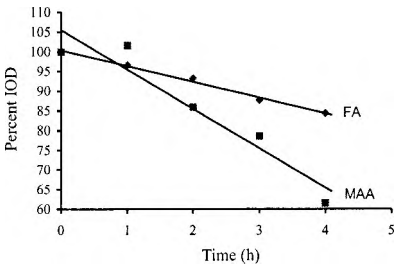

Fig. 4. Influence of prolonged post-hydrolysis washes in distilled water at room temperature on Feulgen stainability of *Pisum sativum* meristems, fixed either with MAA or 4% buffered formaldehyde for 1.5 h. Integrated optical density (IOD) is expressed as percent of dye content after a 5 min wash. Each data point is the mean value of two slides (meristems) of which 10 telophases and 10 prophases were measured. Regressions for formaldehyde-fixed (FA) meristems conform to $y = -4.0168x + 100.44$, $r^2 = 0.9908$, and for methanol acetic acid-fixed (MAA) meristems to $y = -10x + 105.51$, $r^2 = 0.9153$.

and noted a slight, but continuous decrease of dye content from 1 h staining on, so that after 18 to 19 h about 10 percent of dye content are lost ($y = -0.7489x + 100.75$ for the first 11 h, normalised for the theoretical stain content after 1 h as 100.00; $r^2 = 0.8607$). At 8 °C we stained from 1 to 36 h. After 1 h at least 98% of staining have been achieved, a maximum was observed after 5 h, and the regression line was almost horizontal so that even after 36 h 98 percent of maximum staining were observed ($y = -0.0662x + 100.07$, normalised for the theoretical stain content after 1 h as 100.00; $r^2 = 0.097$). This observations would indicate, that at room temperature staining should not be extended much over 1 h, when the material shows good penetrability such as root tips. At cool staining temperatures around 8 °C as typical for refrigerators, staining time is less critical and may be extended over night. Further test are desirable, however.

Interestingly these data show among others, that the staining process restores the stability of DNA. We suppose, that this stabilisation is effected by linking DNA fragments together by dye molecules. If this would not apply, a strong progressive loss of DNA fragments and stainability would occur during the staining process (such as in water after hydrolysis), but this is not observed.

Wash in SO_2 water after staining

The effect of washes in SO_2 water at room temperature after staining was tested from 0.5 h (considered to be the minimum time to avoid plasmatic background staining) to 11 h. We noted practically no dye loss up to 3 h, but then a gradual decrease occurred down to 90% of the original value after 11 h (data not shown). After SO_2 water application usually a short wash in distilled water follows.

Softening in 45% acetic acid

The role of 45% acetic acid is to soften the tissue and to act as a squashing medium. We checked the effect on stain content of nuclei after acetic acid application for 5 min, and 1 to 4 h. We noted a loss of almost 2% stain content per hour (Fig. 5).

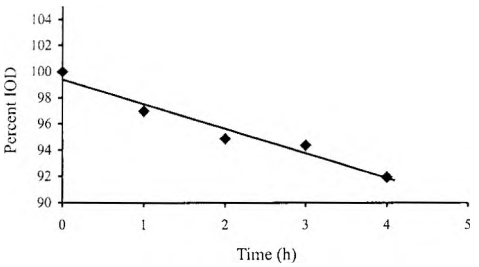

Fig. 5. Influence of prolonged softening of Feulgen stained *Pisum sativum* root tip meristems in 45% acetic acid at room temperature on nuclear dye content. Integrated optical density (IOD) is expressed as percent of dye content after 5 min softening. Each data point is the mean value of two slides (meristems) of which 10 telophases and 10 prophases were measured. Regression conforms to $y = -1.879x + 99.372$, $r^2 = 0.9565$.

Preparation of slides

After making squashes, we use to remove the cover slip over a cold plate, transfer the frozen slides to 96% ethanol and after a few minutes air-dry the slides. The effect of standing in ethanol at room temperature in the dark was investigated over 46 h. Three slides served as control and 3 slides were kept in ethanol and were removed only for measurement. Five particular nuclei per test and control slide were measured 3 times after 1, 4, 16, 26 and 46 h. After 26 h practically no loss of absorbance was recognisable, but after 46 h 2.5% absorbance was lost (details not shown).

Storage of slides

The effect of prolonged storage of slides was tested in formaldehyde-fixed *Glycine max*, *Zea mays*, and *Hordeum vulgare* with *Pisum sativum* as standard (Tab. 3). The slides had been measured first shortly after preparation and another time 41 to 46 weeks later after having been stored in the dark. The nuclei measured were telo- and prophases and both times randomly selected. There was a decay in dye content in the range of 5% which was statistically significant. The more or less parallel loss of IOD in test and standard species resulted in only little relative change in measured DNA content of the test species (Tab. 3).

Discussion

Thanks to the availability of new image analysis systems, that are suitable for rapid quantitative nuclear DNA content measurements, the Feulgen method will probably gain importance especially in the botanical sciences and – as can be foreseen – in developing countries, where the acquisition and maintenance of flow cytometers is difficult. However, perusal of the literature on DNA amounts in plants (BENNETT et al. 2000) reveals frequent discrepancies in data from different laboratories, and it is likely, that many of these incongruities are caused by different experimental procedures. It is highly desirable, that optimised procedures that are generally agreed will be used in future, so that faulty data are minimised. The conclusions from our examinations presented here and also from observations from the past, that are not included in this study, may be summarised as follows.

Tab. 3. Effect of prolonged storage of Feulgen stained slides of various test species and *Pisum sativum* cv. Kleine Rheinländerin as standard. Four tests were conducted. In each test, three slides of test and standard species were stained (10 telophase and 10 prophase nuclei each), measured, stored in the dark, and re-measured (*) after weeks. All re-measured slides were statistically lower at least at $p < 0.05$. Within each test the data are normalised taking the non-aged *Pisum sativum* mean as 1.000.

	Telophases × 2			Prophases			Dye retained	Test species/
Storage time	Mean	SD (n=3)	CV_{in}	Mean	SD (n=3)	CV_{in}		*P. sativum*
Test 1 (*46 weeks)								
G. max	0.279	0.002	2.35	0.276	0.002	2.10	1.000	0.278
*G. max**	0.267	0.008	2.31	0.267	0.004	2.37	0.963	0.281
P. sativum	0.996	0.011	1.72	1.004	0.010	1.13	1.000	
*P. sativum**	0.947	0.014	1.70	0.956	0.022	1.54	0.951	
Test 2 (*43 weeks)								
Z. mays	0.557	0.004	1.73	0.568	0.003	1.40	1.000	0.562
*Z. mays**	0.525	0.011	2.31	0.537	0.014	2.28	0.944	0.552
P. sativum	0.998	0.015	2.41	1.002	0.014	1.47	1.000	
*P. sativum**	0.956	0.013	2.54	0.966	0.009	1.18	0.982	
Test 3 (*43 weeks)								
H. vulgare	1.069	0.018	2.04	1.093	0.021	1.82	1.000	1.081
*H. vulgare**	1.020	0.017	3.01	1.043	0.009	2.31	0.954	1.072
P. sativum	0.998	0.004	1.37	1.002	0.004	1.16	1.000	
*P. sativum**	0.960	0.016	2.26	0.965	0.019	1.34	0.962	
Test 4 (*41 weeks)								
H. vulgare	1.087	0.007	2.62	1.098	0.009	2.69	1.000	1.093
*H. vulgare**	1.030	0.002	2.21	1.043	0.009	2.62	0.948	1.085
P. sativum	0.991	0.016	1.63	1.009	0.007	1.89	1.000	
*P. sativum**	0.947	0.003	1.53	0.964	0.003	1.90	0.958	

(1) MAA-fixed material may be stored in 96% ethanol at –20 °C for at least 5 years without noticeable DNA loss. It is assumed, that formaldehyde-fixed material remains stable at least as long. Formaldehyde has the disadvantage that the resulting staining intensity with the Feulgen method is dependent on the time, the temperature and the concentration of formaldehyde at which fixation is conducted. This has consequences for the stringency with which internal standardisation has to be performed. It has been emphasised many times, that for inter-specific comparisons (genome size determinations) always biological standard material should be co-processed. With using formaldehyde best practice would include a strict co-fixation of standard and test material to avoid any bias due to the factors mentioned above. Although we would recommend that this be also done with MAA-fixation, here the situation is much less critical due to the high stability of material behaviour irrespective whether non-fixed, fixed for short time, or stored for long time. However, the low temperature of –20 °C is crucial for appropriate storage.

(2) Non-bound formaldehyde as a mono-functional fixing agent must and can be completely removed from the fixed tissue. This is best done with small volumes of MAA, which is much more efficient than water. Residual cytoplasmatic staining can be completely avoided according to our experience with many species.

(3) After a short hydration (which is not necessary, but practical, because the material dives faster) the material is hydrolysed. This is the most sensitive step of the whole procedure. It is essential, that besides HCl molarity temperature is stringently controlled (GREILHUBER and BARANYI 1999). Even today, many authors use either 1 M HCl at 60 °C which makes manipulation and time control difficult, or, if they use 5 M HCl, hydrolyse at unspecified »room temperature« (e.g. suggested by DEITCH et al. 1968), which can shift the hydrolysis optimum drastically. For instance, GREILHUBER and BARANYI (1999) noted that a change in room temperature from 20 °C to 25 °C shifts the hydrolysis optimum from 60 min to 25 min. We suggest to conduct hydrolysis at conditions under which use can be made of a relatively long plateau of optimum staining. This »plateau« is not really a plateau, but a segment of the curve in which very little change in stainability occurs. With 5 M HCl at 20 °C this plateau has a length of about 10 – 15 min, at 10 °C 50 min, and at 0 °C 5 h. The respective optima are at 60 min, 220 min, and 20 h. For most purposes hydrolysis at 20 °C will be in order, but 0 °C are easily controlled (ice water) and a long plateau may by useful in certain situations, for instance when material difficult to be penetrated by the reagents has to be stained. Diffusion speed of H_2O is 1.7-fold retarded at 0 °C *versus* 20 °C, but optimum hydrolysis time of 0 °C *versus* 20 °C is prolonged 20-fold (M. SIAM, pers. communic.). Practical tests on the improvement of tissue penetration by reagents at low temperature, as predicted by physico-chemical considerations, are desirable. Moreover, the utility of this 0 °C hydrolysis has to be evaluated. With formaldehyde-fixed material the plateau is much more extended than with MAA-fixed material and there is little difference in this regard between short-term and long-term stored material. The optimum with 20 °C occurs at 90 min and one has to assume that with the other temperatures the optima are also shifted 1.5-fold compared with MAA-fixed material.

(4) The time of washing after hydrolysis is a relatively sensitive point of the procedure, obviously because DNA fragments are mobilised. Within 4 h in distilled water at room temperature 40% and 16% of dye content are lost with MAA-fixed and formaldehyde-fixed material, respectively. The losses that occur in the first hour should be investigated more closely. The data indicate, that this wash should be conducted at a minimum time just enough to remove most of the HCl from the tissue. Five changes within 2 min and the use of ice water could be advantageous.

(5) Usually 1.5 h at room temperature are recommended as staining time with Schiff´s reagent. Staining time at 20 °C should not be extended beyond that, because a slight but continuous loss of intensity is observed from the first hour of staining on. On the other hand, staining at 8 °C showed near- optimum dye binding even after more than 24 h.

(6) Washes in SO_2 water are necessary to remove Schiff´s reagent in the de-coloured state at which it can be easily removed from the tissue, which is not the case with re-coloured reagent. We found, that the usually recommended 30 min and 3 changes of SO_2 water are often not completely sufficient to remove all reagent. We suggest to extend to 45 min and change at least 6 times (3 times in short intervals at the beginning). Interestingly, the Feulgen-DNA-complex is much more stable than hydrolysed DNA alone.

(7) Softening in 45% acetic acid should be kept at a minimum, say 3 to 5 min, sufficient to penetrate the material. Standard and test material should be squashed in close sequence.

(8) We use to remove cover slips over a cold plate and pass slides 2 min through 96% ethanol before air-drying them. The stain intensity is fairly insensitive against a prolonged

ethanol bath. Slides may be advantageously measured under immersion oil optics without application of a cover slip.

(9) Slides stored in the dark are relatively stable. We expect that within 2 weeks there should be no significant deterioration of stain intensity, and the use of an internal standard should largely cancel out all shifts that may occur even after longer time, say 2 months.

We believe that more research is necessary on practical aspects of the Feulgen reaction, especially in plants. All steps of the procedure are influencing the final result, some more than others. Treatment steps prolonged beyond the optimum or the necessary lead to a decay of staining intensity. This decay probably does not any more proceed in a stoichiometric manner, as certainly factors such as chromatin density differences within the test material and between standard and test material are brought into play.

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