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A rapid method by flow cytometry for estimating persistence of tetraploid perennial ryegrass in pasture mixtures with diploid perennial ryegrass

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Abstract

A simple and rapid method for the estimation of the proportion of diploid and tetraploid perennial ryegrass in a mixed sward is described, based on the measurement of the nuclear DNA content of the leaf tissue cells by flow cytometry. The error of estimation was less than 5 %.

Introduction

The tetraploid cultivars of perennial ryegrass (*Lolium perenne* L.) have some advantages over the diploid cultivars. They are more palatable and less crown rust susceptible than diploids. However, tetraploids are less persistent than diploids. To combine the advantages of both ploidy levels, the mixtures composed on a fifty-fifty seed number base, are recommended to the farmers. Because of the differences in the establishment rate and persistence between diploids and tetraploids, the initially sown fifty-fifty rate may change in time.

The current methods of determining the actual rate in the pasture are based on an individual plant analysis, *i.e.* a microscopic analysis of squashed and stained root tips (Rogiers *et al.* 1988) or flow cytometric analysis of leaf tissue. The estimation of ploidy rate by a flow cytometric analysis of the mixed samples of diploid and tetraploid leaf tissue is theoretically difficult because of: (1) overlapping of the diploid G_2 peak and the tetraploid G_0/G_1 peak, (2) the presence of doublets, triplets *etc.*, (3) a smaller number of tetraploid cells per leaf tissue area or mass unit as a consequence of the greater cell size of tetraploids (De Roo 1968, Wilkins and Sabanci 1990), (4) the inevitable apparatus noise and debris, resulting from disrupted nuclei and non-specific staining of other cell constituents (which seems, from our experience, to be greater when analysing tetraploid grass).

The present study was undertaken to cope with these problems in an empirical way.

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Materials and methods

The leaf samples of one year old grass plants of the known ploidy level were taken 5 d after mowing. Rectangles of 20 mm^2 were cut from these leaves. The length of the rectangle depended on the full width of the leaf. Mixed samples of 25 leaf pieces were composed in all rates from 0 to 25 diploid and 25 to 0 tetraploid parts. The mixed samples (500 mm^2 leaf or ± 70 mg) were chopped with a sharp razor blade in 0.5 ml buffer solution, containing 0.1 M Tris, 2 mM $MgCl_2$, 0.1 M NaCl and 0.1% *Non Idet P40*. Afterwards, 1 ml of the same buffer with the fluorochrome DAPI (2.5 mg l^{-1}) was added. 30 s later the preparations were passed through a nylon filter of 50 µm mesh size and kept on ice until the analysis.

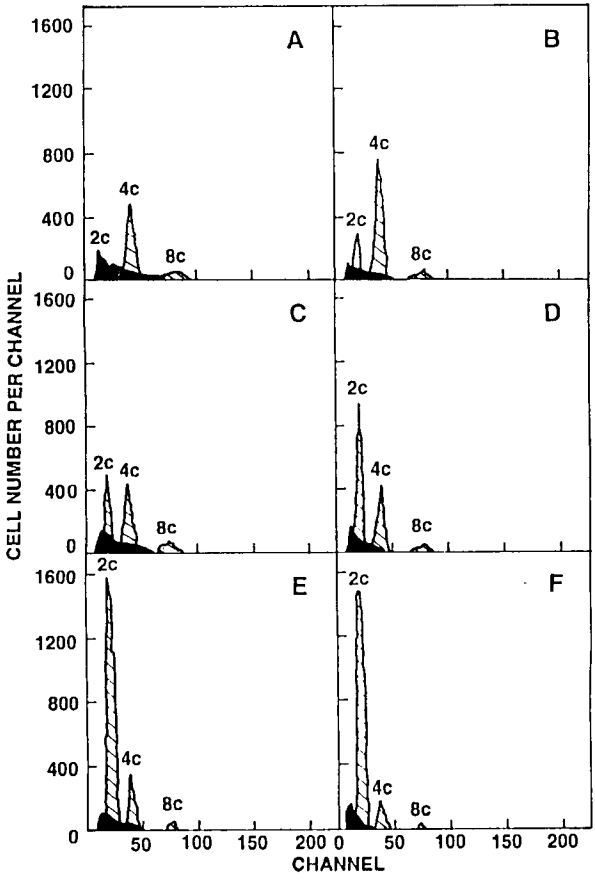

Fig. 1. DNA-histograms from nuclear preparations from leaf mixtures of diploid and tetraploid perennial ryegrass: (*A*) 100 % tetraploid; (*B*) 80 % tetraploid, 20 % diploid; (*C*) 60 % tetraploid, 40 % diploid; (*D*) 40 % tetraploid, 60 % diploid; (*E*) 20 % tetraploid, 80 % diploid; (*F*) 100 % diploid

The flow cytometric analysis was performed by the *Partec Cell Analyzer CA-II*. In each sample 10000 particles were analyzed. The DNA distribution curves were analysed with the *DPAC* (Data Pool Application for *CA-II*) software. This software allows to calculate the surface area of the DNA-peaks, which also contain some background noise. Regressions were calculated with the real ploidy rate as dependent variable and the rate of the area of the diploid G_0/G_1 peak to the total area of all visible peaks as the independent variable.

Results

Fig. 1 shows some of the histograms of different diploid/tetraploid mixtures. Shaded areas represent background noise and debris. Tetraploid G_0/G_1 peaks were wider than diploid G_0/G_1 peaks. Although the same amount of particles in all mixtures were counted, the mixtures, predominated by tetraploid components, showed smaller peaks and consequently relative more noise. In the pure diploid sample a peak was observed on the channel of the G2M tetraploid peak. This indicates an endopolyploid state. The *c.v.*-value (coefficient of variation, defined as standard deviation of a peak, expressed as percentage of the mean peak channel) both for the diploid and tetraploid G_0/G_1 peaks ranged from 5 to 10 %.

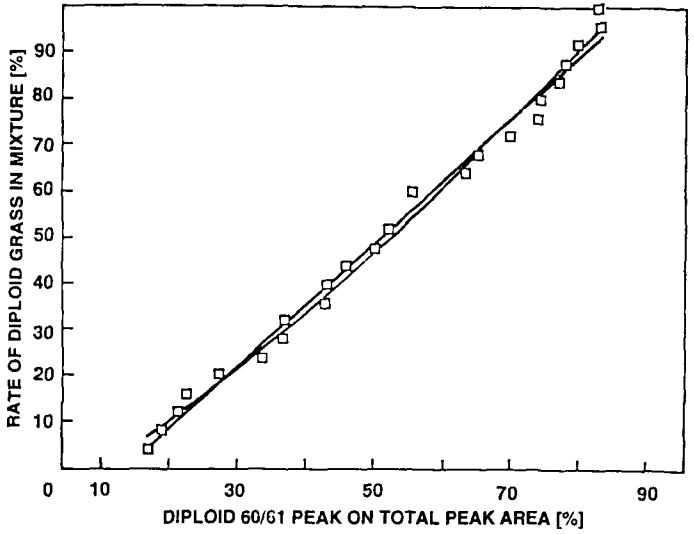

Fig. 2. Regression of the real proportion of diploid perennial ryegrass in a mixture (y) on the ratio of the diploid G_0/G_1 peak area to the total peak area (x)
linear model: $y = -18.6 + 1.4\ x$ $R^2 = 99.1$ $SE = 3.0$
quadratic model: $y = -11.9 + 1.0\ x + 0.003\ x^2$ $R^2 = 99.2$ $SE = 2.8$

The results of the regression analysis are shown in Fig. 2. The pure tetraploid sample was not involved in the calculation because of the absence of a diploid G_0/G_1

peak. A simple linear regression explained already more than 99 % of the total variance. The standard error of estimation (*SE*) for the linear model was less than 3 %. The adjustment to a quadratic curve was significant and improved slightly the R^2 coefficient and the *SE*.

Discussion

Samples were taken from the early regrowth of one year old plants. So the regrowth of the juvenile as well as of the one year old shoots was involved. This may explain rather high width and *c.v.*-values of the peaks and the presence of endopolyploid cells. De Laat *et al.* (1987) found *c.v.*-values of less than 1.5 % in peaks of preparations from true leaves of sugarbeet seedlings.

The different physiological state and age of the grass may influence the distribution of the nuclei over the cell-cycle stages as well and, as a consequence, the relative height of the G_0/G_1 and G_2 peaks may vary. Nevertheless the error of estimation for the calculated model is rather low.

Not only the sampled material but also the analyst and the apparatus may cause a variation in the observed values. Therefore gauging the apparatus at regular times is recommended. This may be accurately done on the basis of a few mixed samples of known composition. The standard error of the estimation of the above-mentioned 25 mixed samples for a linear model, calculated out of 5 samples (20, 40, 60, 80 and 100 % diploid leaf material) was 3.8 %, which was only slightly higher than the 3.0 % of the model calculated out of all 25 samples.

The preparation and analysis, both microscopic and flow cytometric, of 100 individual plants takes 8 working-hours. 20 mixtures of 25 plants, that makes 500 plants, and a standard series of 5 known mixtures may be prepared and analysed in 8 working-hours as well.

Conclusion

The described method allows a prediction of the proportion of diploid and tetraploid perennial ryegrass in a mixed sward with an estimation error less than 5 %. Moreover this method is five times faster than any current method. The gain of time may be applied to sample more plants and/or more frequently.

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