

Single Corn Kernel Wide — Line MMR Oil Analysis for Breeding Purpose

M. C. C. WILMERS, C. RETTORI, H. VARGAS, G. E. BARBERIS
Instituto de Física Gleb Wataghin, Universidade Estadual de Campinas,

and

W. J. DA SILVA
Instituto de Biologia, Universidade Estadual de Campinas, C.P. 1170, 13100Campinas SP

Recebido em 22 de Novembro de 1977

The Wide-Line NMR technique was used to determine the oil content in single corn seeds. Using distinct radio frequency (RF) power, a systematic work was done in kernels with about 10% of moisture, and also in artificially dried seeds with approximately 5% of moisture. For non-dried seeds NMR spectra showed clearly the presence of three resonances with different RF saturation factor. For dried seeds, the oil concentration determined by NMR was highly correlated ($r = 0,997$) with that determined by a gravimetric method. The highest discrepancy between the two methods was found to be about 1.3%. When relative measurements are required as in the case of single kernel for recurrent selection program, precision in the individual selected kernel will be about 2.5%. Applying this technique, a first cycle of recurrent selection using S_1 lines for low and high oil content was performed in an open pollinated variety. Gain from selection was 12.0 and 14.1% in the populations for high and low oil contents, respectively.

Utilizamos a técnica da Ressonância Magnética Nuclear de onda contínua, para determinar o conteúdo de óleo em sementes individuais de milho. Usando distintas potências de rádio frequência (RF) foi feito um trabalho sistemático em grãos com cerca de 10% de umidade, e também em sementes artificialmente dessecadas, com aproximadamente 5% de umidade. Para as não dessecadas, o espectro da RMN apresenta claramente três ressonâncias com diferentes fatores de saturação de RF. Para as dessecadas, a concentração de Óleo determinada por RMN, está altamen-

te correlacionada ($r = 0,997$) com a determinada pelo método gravimétrico. A maior discrepância entre os dois métodos, foi de cerca de 1,3%. Quando fazemos medidas relativas, como no caso de programas de seleção recorrente com grãos de milho individuais, a precisão em cada grão selecionado é da ordem de 2,5%. Aplicando esta técnica, foi feito um primeiro ciclo de seleção recorrente usando S_1 linhas para alto e baixo conteúdo de Óleo, numa variedade de polinização aberta. O ganho de seleção foi de 12,0 e 14,1% para as populações de alto e baixo teor de Óleo, respectivamente.

1. INTRODUCTION

Proton magnetic resonance or Nuclear Magnetic Resonance (NMR) of the hydrogens atoms has been used lately as important tool for individual and non-destructive analysis of oil content in many different living seeds^{1,2,3,4,5,6} for breeding purpose. Accuracy, precision and short time experiment are the most important requirements to be fulfilled in any plant breeding program. Besides that the same selected seeds have to be planted in the breeding nursery in order to insure the maximum progress in oil content per generation^{7,8}.

There are basically three different ways of using the NMR technique, 1) the Wide-Line NMR as was shown by Conway and Earle (1963), Alexander *et al.* (1967), Collins *et al.* (1967) and Anderson (1971); 2) the Pulsed NMR⁵, and 3) High Resolution NMR⁶. All of them are good enough to guarantee genetic gains to plant breeders.

In our case, however we want to report Wide-Line NMR experiments, where special care was taken in order to insure good accuracy (absolute oil concentration determination), and precision (good reproducibility), although fast measurements should not be expected with this method^{5,7}. Using the procedure developed in our laboratory we performed one cycle of recurrent selection for low and high oil content in a corn variety using NMR single kernel analysis.

2. EXPERIMENTAL PROCEDURE

NMR studies

The Wide-Line NMR equipment used in these experiments consists of a commercial Varian WL - 210 unit with variable frequency (3.0 - 35 MHz) stabilized within $1/10^6$ and an RF power output of 100 mw stabilized within $1/10^4$, which could be attenuated in steps of 20 db and continuously.

The signal is integrated in order to get a number proportional to the number of resonating spins⁸ which in turn will give a number proportional to the oil content. The integration process is carried on by a Varian 620 L analogic digital computer running a specially adapted software program⁹. The whole system is working in a similar way to that used by Anderson⁴, except for the small amplitude of modulation field.

The stability of the whole systems was tested by monitoring the NMR resonance of a standard 20% deuterated water sample. The test was done at different levels of a) RF power levels, b) NMR frequencies and c) amplitude of modulation field. The reproducibility of the absorption derivative, as well as the integration process, was better than 1% for any set of parameters mentioned above.

Since the intensity of the signal depends on the relative orientation of the corn seed respect to the RF coil⁵, special care was taken to locate the sample at the position where the narrowest and strongest signal were obtained. When the RF coil was at the maximum field homogeneity it was found that small changes (less than 10%) were detected for different positions of the corn seed when the germ was along the coil axis. In any case it was fairly easy to locate the sample at the optimum position. Again reproducibility on the same sample was better than 3%.

The RF power level was monitored by a microamperimeter which gives the RF current on the transmitter coil. This current gives a relative measurement of the power incident on the sample.

Since in this work we do not need absolute measurements of that power, we shall indicate the relative power in arbitrary units (μA) in the whole work. Every time that the RF power was changed the whole equipment was turned up. No detuned effects were observed by changing samples. The RF frequency used was always around 30 MHz (7035 Gauss).

Corn seeds for oil evaluation, were dried in a conventional air forced oven at $40^{\circ}C$, during seven days. In this way the moisture level was kept below 5% and the seeds were all alive. Seeds were maintained in sealed pyrex tubes to avoid possible rehidratation.

The gravimetric method⁹ to determine oil content was done on a Goldfisher equipment using a ground sample of five seeds. This mixture was continuously washed out with petroleum ether during four hours. The oil and solvent were collected in a previous weighted tube. The solvent was evaporated and the mass of oil determined by weight difference. The reproducibility of this method for groups of twenty five seeds of the same sample was found to be about 2%.

Selection Program

Using the described procedure it was started a selection program to improve oil content of a dent tropical synthetic designated Maya, obtained from the Instituto Agronômico de Campinas.

In the 1975 season was planted at the Experimental Area of the Universidade Estadual de Campinas ear-to-row progenies selected from agronomical desirable plants of the 1974 nursery. The 50 progenies showing better phenotypic aspect were selected by flowering time and the superior plants within the row were marked and selfpollinated. From the best plant of each selected 50 row, it was taken the selfpollinated ear for NMR analysis. Two kernels were taken from the central part of each ear for oil evaluation. In these 100 kernels, measurements were performed using our non-destructive method to start a selection program. From the highest and lowest 10% of the distribution it was made two composites: one for high and other for low oil content. The two populations were

planted separately and at the flowering time was performed free recombinations within the groups. At the same time a balanced composite was made out of the 50 ears to be used as checker for progress evaluation in further generations.

3. RESULTS AND DISCUSSION

Fig. 1 shows typical NMR spectra of a non-dried corn kernel with approximately 10% moisture. It can be seen that the spectra presented a strong dependence on the RF power level.

In Fig. 1-a it is possible to distinguish three resonances with approximately similar gyromagnetic ratios¹⁰, but with different line widths. At resonance the RF power absorbed by the sample can be written as^{8,10}

$$P(H) = \omega \frac{H^2}{1} \chi_0 / |1 + \gamma^2 (H - H_0)^2 \frac{T_1^2}{T_2} + \gamma^2 H^2 T_1 T_2| \quad (1)$$

where γ is the gyromagnetic ratio, H_1 the RF magnetic field, H_0 the resonance static magnetic field, χ_0 the static nuclear magnetic susceptibility and T_1 and T_2 the spin-lattice and spin-spin relaxation times respectively⁸.

The three different resonances observed in fig.1-a suggest the presence of hydrogen nuclei bounded to different surroundings, which will cause a) different chemical shifts⁶, b) different spin-spin relaxation time (T_2)⁸, and c) different spin-lattice relaxation time (T_1).⁸ Feature a will give different resonant field, b different linewidth (γT_2)⁻¹ and c different saturation factor $\gamma^2 \frac{H^2 T_1 T_2}{1}$ i.e. the level of RF power needed for saturation will be different for each of them.

In order to test this qualitative behavior we increased the RF power. Fig. 1-b actually shows that at $I_{RF} = 200 \mu A$ the narrowest resonance is already saturated. Fig. 1-c shows that at $I_{RF} = 400 \mu A$ the two narrower resonances are now saturated and the broader is the one that has the highest saturation factor $\gamma^2 \frac{H^2 T_1 T_2}{1}$. Knowing we were dealing with three different proton resonances, the next step was to try to identify them.

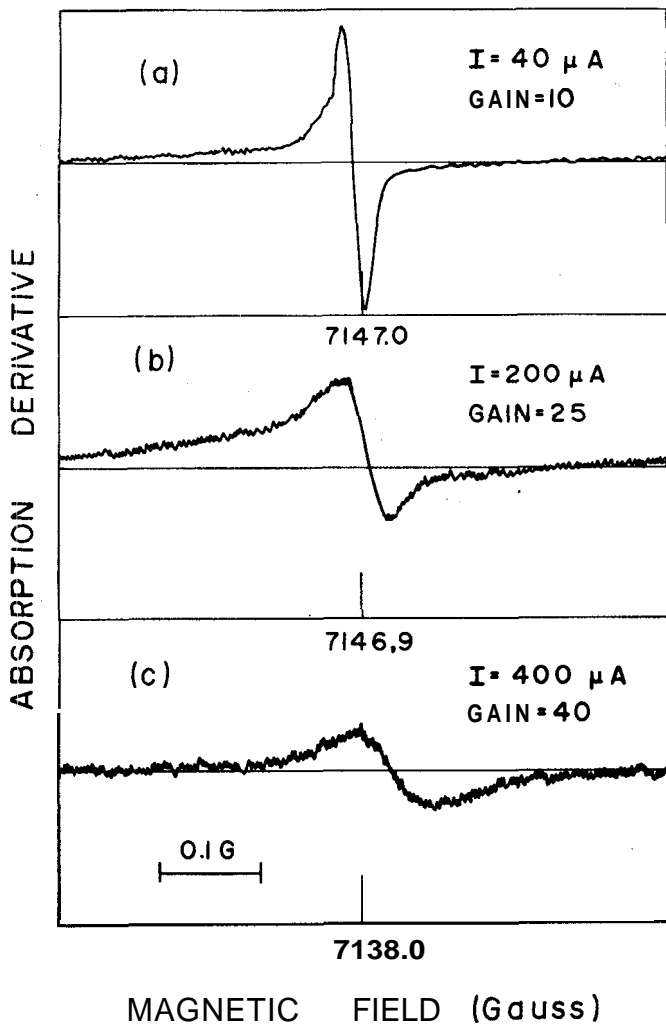


Fig. 1 - Power dependence of the NMR signal for a non-dried corn seed with approximately 10% moisture.

Fig. 2 shows the resonance of the same corn kernel after been dried for seven days at 40°C. The seed weight reduction was about 5 to 6% what means that moisture left in the kernel was about 4 to 5%. The spectra shows clearly the absence of a broader resonance at any RF power level. Hence we attribute this resonance to the hydrogens nuclei of the water

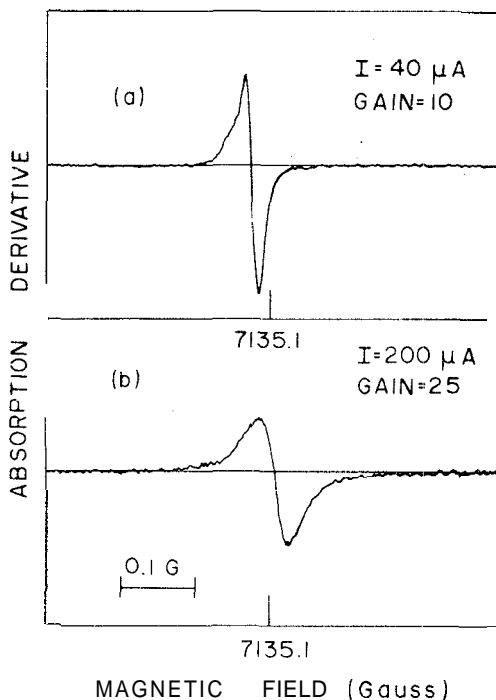


Fig. 2 - Power dependence of the NMR signal for a corn seed after dried during seven days at $40^{\circ}C$.

molecules. Since the water resonance is broader than the resonance of pure liquid water we believe that the water molecules in the seed might be slightly bounded to their environment.

All the hydrogen atoms in the solid phase, like those of proteins and carbohydrates will give resonances too broad to be detected since they have low mobility and the effects of spin-spin interactions (short T_2) will not be motionally narrowed⁸. Thus we concluded that the two narrow resonances observed in fig. 2-a and 2-b should come from different groups of hydrogen nuclei of the corn oil molecules. Actually the expected shift corresponding to the splitting of 30 mG, observed between the two resonances of fig. 2-a, correspond to the chemical shift between $(CH_2)_n$ and $=C^H-C^H=0$ measured by Conway and Johnson in high resolution NMR experiments⁶. Also their relative intensity are quite similar.

Further evidence that the two narrower lines come from oil component is given by the fact that the spectra of the germ alone reproduce this two resonances and the one attributed to the water molecules was only found into the endosperm (see fig. 3). This is apparently so because oil is mainly distributed in the corn germ.

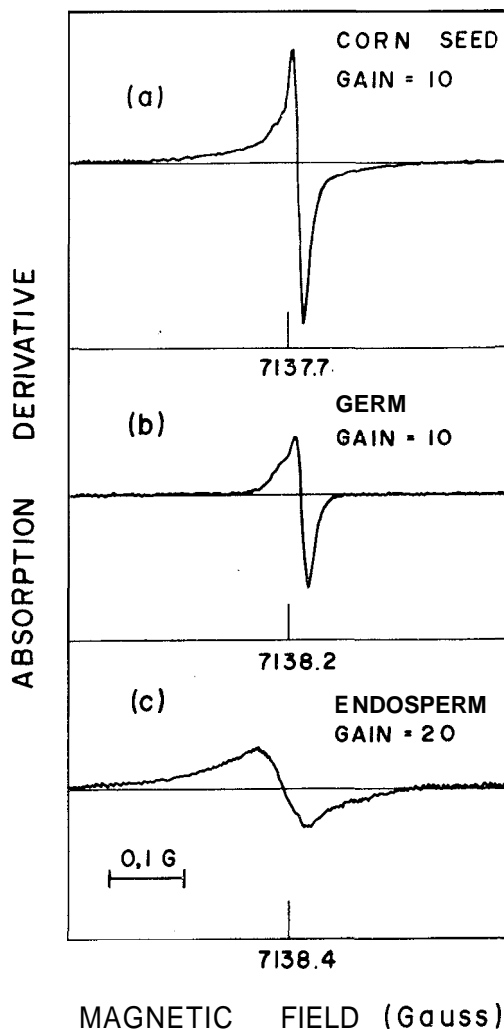


Fig. 3 - Proton RMR resonances for different parts of a corn seed. a) Resonance for the whole seed; b) Resonance for the germ; c) Resonance for the endosperm.

In order to see the individual contribution to the total resonance intensity at different RF power levels, we have integrated twice the spectra of a natural (non-dried) and of a dried seed. Fig. 4 shows the experimental saturation curve corresponding to the superposition of three resonances. Each individual resonance has a saturation curve given by their appropriated relaxation times T_1 and T_2 , according to expression (1).

At low RF power level ($I_{RF} = 30 \mu A$) the total intensity is mainly given by the oil components and at high RF power ($I_{RF} = 300 \mu A$) by the water component. This will in principle suggest that both oil and moisture concentration can be determined on non-dried single corn kernel by just measuring the total resonance intensity at different RF power levels.

In our particular case the signal to noise ratio at low and high RF power are not good enough to give the required precision and accuracy for the measurements. Therefore we were forced to work at intermediate RF power ($30 < I_{RF} < 60 \mu A$), where the resonance in the natural seed will have a mixture of the two components, oil and moisture. Hence an accurate oil determination can be given only if the corn kernel is dried at moisture levels lower than 5% (see fig. 4 curve b and c).

A systematic individual NMR analysis on 80 corn seeds has been done. The seeds were part of eight groups of ten kernels each. The seeds of the same group had similar genetic background. The absolute concentration has been obtained by direct comparison of the intensity of the NMR resonance of each seed with the one detected in a standard corn liquid oil sample. The reproducibility in different runs of the same seed and standard was always better than 3%. Therefore we can say that our accuracy and precision is about 5% for the absolute oil concentration which will give a maximum error of about 1 mg in the oil determination of each single corn kernel.

Fig. 5 shows a typical spectra of the corn liquid oil standards. This is completely similar to the spectra obtained on dried corn seed (fig. 2-a).

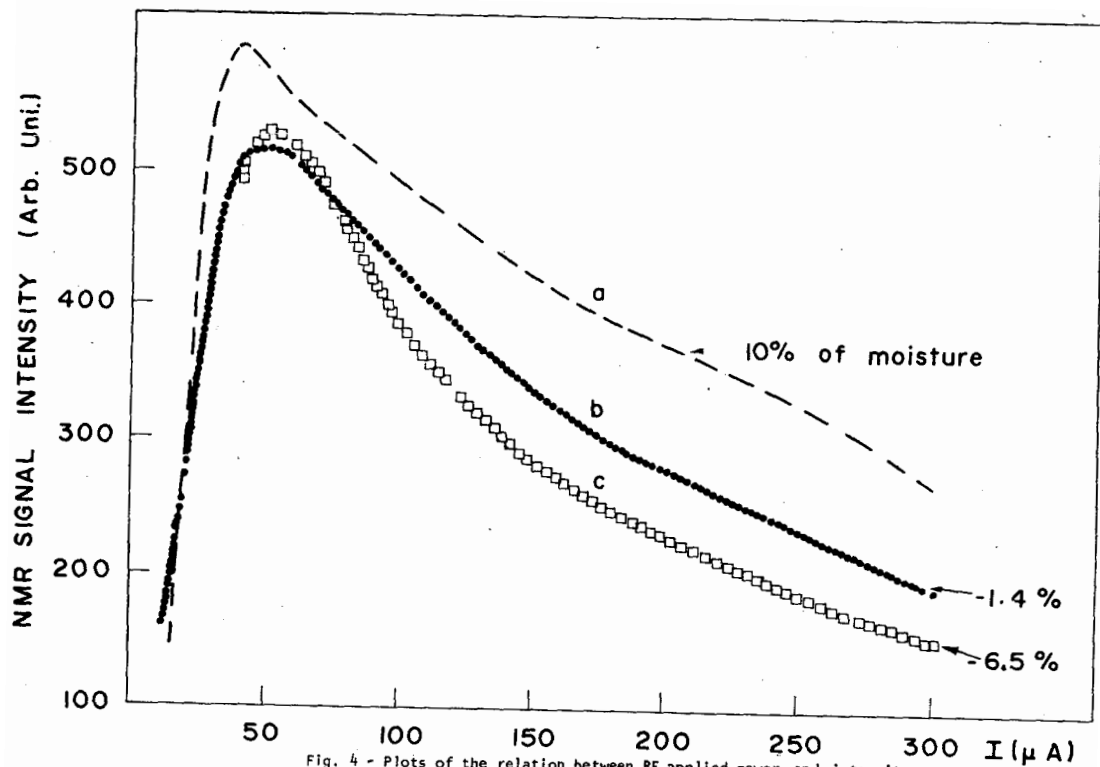


Fig. 4 - Plots of the relation between RF applied power and intensity of the NMR signal. The square dots correspond to a dried seed to 3.5% of moisture; the round dots to a 8.6% moisture seed, and the dashed line to natural (10% moisture) occurring seed.

ABSORPTION DERIVATIVE

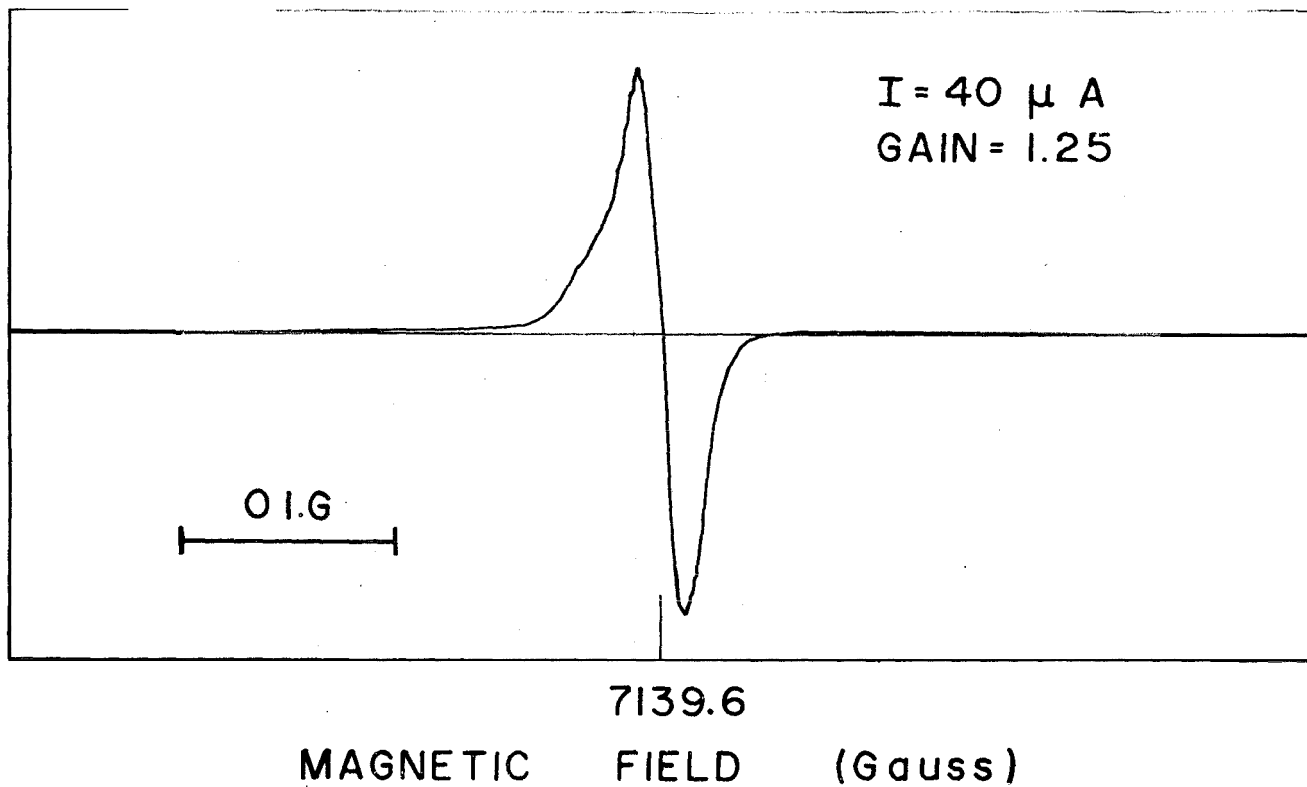


Fig. 5 - NMR signal obtained from liquid corn oil.

In order to study the correlation between the NMR method and the gravimetric procedure, eight different corn germplasm groups were analysed by the gravimetric method for oil content.

Fig.6 shows a correlation coefficient of 0.997 between the two methods. The gravimetric method gave an error of approximately 5% and the error bar for the NMR analysis is also about 5%. As the two methods are hi-

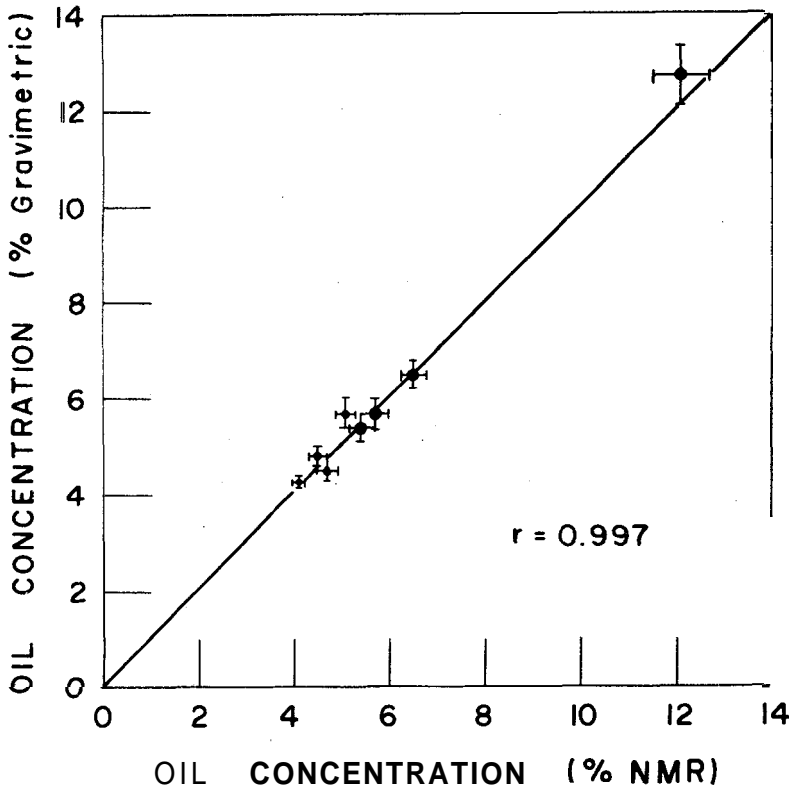


Fig. 6 - Correlation between oil concentration measurements. The experimental points represented were obtained by single seed NMR measurements and gravimetric measurements for groups of 10 seeds. The horizontal values are the mean value obtained in NMR analysis for the same ten seeds.

ghly correlated and the regression coefficient of oil percentage by the gravimetric method on the NMR procedure is close to 1.0 and the errors are comparable we believe this method is quite promising for plant breeding purpose.

In the selection program, the synthetic Maya was chosen by revealing the highest oil content (5.1%), among 12 of our most yielding commercial varieties. Results on oil content of the 100 kernels are shown on fig. 7. Data ranged from 1.9 to 7.2% with an average of 5.08%. Selection was performed on basis of progene mean $\pm 1.76 \sigma$, giving truncation points of 6.3%, for the upper limit and 4.0% for the lower group. This represented a selection intensity of approximately 10% for each set. The two groups are the basic germplasm for a recurrent selection using S_1 lines for high and low oil content.

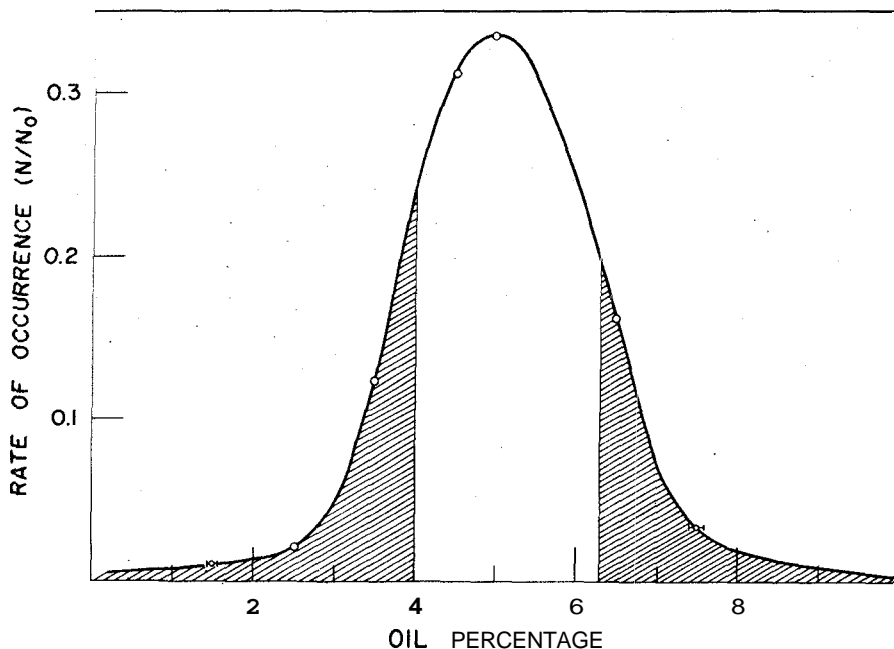


Fig. 7 - Distribution of oil content of 100 kernels analysed by NMR technique. The dashed zone corresponds to the selected high and low concentration kernels.

Recombination within the two groups gave a gain of 12.0% for the high oil population whereas the progress from selection in the low oil population was 14.1%.

The genetic progress obtained in this program gives the final **evidence** for the suitability of the suggested NMR method for breeding purpose. .

This research was supported in part by research grants from PIG - CNPq-
FAPESP - FINEP

REFERENCES

1. Conway, T.F. and Earle F. R., *J.Amer.Oil Chem.Soc.* 40, 265 (1963).
2. Alexander, D.E., Silvela S. L., Collins, F.I., and Rodgers R., *J. Amer. Oil Chem. Soc.* 44, 555 (1967).
3. Collins, F.I., Alexander, D.E., Rodgers, R.C., Silvela, S.L., *J.Amer. Oil Chem. Soc.* 44, 708 (1967).
4. Anderson, L.O., *J. Amer. Oil Chem. Soc.* 48, 47 (1971).
5. Tiwarii, P.N., Rajan, Gambhir and T.S., *J. Amer. Oil Chem. Soc.* 51, 104 (1974).
6. Conway, T. F. and Johnson, L. F., *Science* 164, 827 (1969).
7. Zupancic, I., Vrscaj, I.S., Porok, J., Levstek, I., Erzen, V., Blinc, R., Pausak, S., Ehrenberg, L., and Dumanovic, J., *Acta Chem. Scand.* 21, 1664 (1967).
8. Abragam, A. *The principles of Nuclear Magnetism.* Oxford Univ. 1961.
9. Wilmers, M.C.C. MS Thesis - UNICAMP (1977) unpublished).
10. Rollwitz, WL and Persyn, GA, *J. Amer. Oil Chem.Soc.* 48, 59 (1971).