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TITLE

In-vitro mutation breeding technology in maize

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FINAL REPORT

Contract number	:	4523/TC
Title of Project	:	In vitro mutation breeding technology in maize
Institute	:	Maize Research Institute, Trnava, Czechoslovakia
Chief scientific investigator :		Milan Neštický
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Background of Project

At present breeders use only very small part of already created maize inbreds for hybrid production. These inbreds have good combining ability and good agronomic characters. It results in narrow genetic base of hybrids and a lot of recent hybrids are more or less related to each other. There is a risk of vulnerability of these hybrids by any pest or disease. Also response of hybrids to unfavourable climatic conditions is almost the same. In both cases it could cause callamity in maize production.

There are some genetic resources for improving combining ability in the world maize collection, but to introduce such a complicated character as combining ability into another genotypes is time consuming and breeders are not willing to spend time in this work. Moreover, there are other limitations /vegetation period, sensitivity to drylength etc./ which make results of work unclear.
The main goal of this Project is to assess induced and somaclonal variabilityas a mean for increasing genetic variability of maize.

Description of research work carried out

Research objectives of the project were oriented on three directions

1. Evaluation of somaclonal and induced variability in plants regenerated from *in vitro* cultures.
2. Expression of somaclonal variability in heterozygous stage.
3. Improvement of somatic embryogenesis of different genotypes of maize.

1. Evaluation of somaclonal and induced variability was carried out in field experiment. The inbred CHI 31 possessing a high *in vitro* capacity of somatic embryo formation was used as experimental material.
   Following variants were used in the experiment
   ES0 - plants derived from seeds /check/
   ES1 - immature cobs /12 days after selfpollination/were exposed to 5 Gy of ^{60}Co gama radiation and grown to maturity. At maturity caryopses were harvested as M_1 material.
   ES2 - the same procedure as ES1 except for treatment. This variant was irradiated with 10 Gy ^{60}Co gamma radiation.
   In variants ES1 and ES2 next generation designed as M_2 generation was evaluated.
   ET0 - immature zygotic embryos /size 1,2 - 1,5 mm/ were excised from caryopses and cultivated on N_6 nutrient medium according to NOVÁK et al. /1983/. Plants regenerating from somatic embryos were transplanted into soil and designed as R_1 material. The standard experimental protocol for somatic embryo formation is given in Figure 1. Progenies of R_1 generation designed as R_2 generation were evaluated in the experiment.
   ET1 - the same procedure as ET0 but before cultivation *in vitro* immature zygotic embryos were treated with 5 Gy of ^{60}Co gamma radiation. Regenerated plants were considered as M_1R_1 material.
   ET2 - the same procedure as ET1 but embryos were treated with 10 Gy of ^{60}Co gamma irradiation.

The experimental protocol for assessment somaclonal and induced genetic variability is given in Figure 2. Progenies of ET1 and ET2 variants designed as M_2R_2 generation were evaluated in the experiment.
All above mentioned biological materials were created in the Agricultural Biotechnology Laboratory in Seiberedorf and seeds were delivered to the Maize Research Institute in Trnava. Totally 12 883 plants were planted at the experimental base of the Maize Research Institute.

During vegetation period and after harvest following trait, were evaluated:

- chlorophyll changes
- morphological alternations including dwarf types, multistem types, etc.
- lethality of seedlings
- time of flowering
- plant height, ear height
- ear length
- ear diameter
- number of rows in one ear
- number of kernels in one row

2. Expression of somaclonal variation on heterozygous level was evaluated in hybrids from top-cross 20 progenies from regenerated plants of the inbred line CHI 31 experimental variant ET0/ were crossed with tester Bu $8Ro_2$. Hybrids from top-cross were tested in trial with four replications, plot consisted of two rows, 23 hills in one row, 70x25 cm spacing. At maturity time following traits were evaluated
   - plant height
   - ear position
   - yield

3. Improvement of somatic embryogenesis of different maize genotypes was studied in two genetic systems:

a/ high embryogenic inbred line CHI 31 was crossed with multitester 76-1819-3 in direct and reciprocal directions. Hybrids F_1 was back-crossed to both parents to B_1 generation.

b/ screening of 17 genotypes for expression of embryogenic callus formation and regeneration was carried out. 5 inbreds from this set were chosen to back-cross with inbred CHI 31 as a donor of embryogenic callus. Inbreds were evaluated after three back-crosses. The used genotypes in this experiment are listed in Tab.3 and Tab. 4.

Obtained results

1. Evaluation of somaclonal and induced genetic variability

Table 1 gives the frequency of morphological and chlorophyll variants segregating in the M_2, P_2 and M_2R_2 generation. In vitro irradiation of zygotic embryos before harvesting of seeds induced 0,22 and 0,24 percent of chlorophyll variants, and 3,43 and 2,77 percent of morphological variants segregating in the M_2 generation after exposures to 5 and 10 Gy, respectively. The frequency of both chlorophyll and morphological variants /1,94 and 5,39 respectively/ obtained in the R_2 generation was significantly exceeding the variant frequencies derived from the M_2 generation. The combination of explant iradiation and in vitro regeneration was most effective for increasing the variant frequencies. The frequency of early flowering variants was increased to 2,89 and 4,14 % in M_2R_2 generation compared with 0,5 % in the R_2 and 0,42 % in M_2 generation. The same is true for multistem plants. The frequency of multistem plants continously increases from the M_2 generation /0,29%/ to the R_2 generation /0,34%/ with the highest value in the M_2R_2 generation /0,96 %/.

Radiation induced and somaclonal variation exerted a similar spectrum of chlorophyll and morphological variants. The most frequent chlorophyll variations were virescent or pale green seedlings and plants with white or yellowish leaf stripes. Morphological variants in the M_2, R_2 and M_2R_2 generation were brachytic with shortened internodes, erect narrow leaves, wrinkled leaves and dwarfs. Most of morphological variants had reduced tassel what prevented to selfpollinate plants and to investigate genetical nature of variants. Plants with 2-3 stalks occured in all experimental variants. In one case of two-stem plants one stem was albino and second green. Albino plants died in stage of four leaves. In all experimental variants occured plants with decreased ability to grow which can be named semilethal variants. Plants of this variant grew to stage 5-6 leaves. Then necrosis on leaves occured and plants died.

In Table 1 are presented mostly qualitative variants. From the point of view of breeding quantitative characters are more interesting because most of economically important characters are controlled by polygenic systems. Therefore, we studied also variation of experimental variants on following quantitative characters:

plant height
ear length , ear position
ear diameter
number of rows in one row
number of kernels in one row

Results of study are given in Figures 3,4,5,6, 7 and 8. From the results it is clear that variation expressed in all studied characters and in all experimental variants. In all characters except ear length the check reached the highest value. The R_2 generation /experimental variant ET0/ had in all studied characters lower values in comparison with the M_2 generation /experimental variants ES1 and ES2/. Generally can be stated that average value of characters continously decreased in dependence on treatment and combi-

nation of explant irradiation and *in vitro* regeneration.

2. Expression of somaclonal variability in heterozygous stage

In a system of top-cross, twenty regenerates were crossed with the tester line Bu 8 R o_2 to find out an expression of somaclonal variation at a hybrid level. The hybrids from top-cross were tested for plant height, ear position and yield. The results from this test are given in Tab.2

Plant height. The check hybrid reached an average plant height of 171 cm. The range of this character in tested hybrids was from 161 to 185 cm. A comparison by t-test showed significant differences in plant height for six hybrids /33%/. It can be stated that the combining ability of regenerates included in hybrids No. 3, 9, 10, 15, 16, 18 is changed in comparison to the original line.

Ear position. The range of variability in the tested set of hybrids for this character was 12 cm /from 56 to 68/. The mean of the check hybrid was 66 cm. In this character six tested hybrids /No. 1, 5, 6, 13, 17 and 20/ also differed statistically from the check. For a comparison of each hybrid to those in the tested set we used Duncan's test. We found out that 27 % of pair hybrid comparisons were statistically significant.

Grain yield. As in this character we could not use t-test, we used LSD as a criterion for the statistical significance of differences. The check hybrid had an average yield of 7,351 $t.ha^{-1}$. The range of variability in the tested set of hybrids was 2,535 $t.ha^{-1}$ /from 6,323 to 8,658 $t.ha^{-1}$/. At a level of 0,05 probability, 14 hybrids were different from the check. The best performing hybrid was No. 20, which outyielded the check as well as the other hybrids.

The results of testing the hybrids from top-cross show that somaclonal variation can be expressed not only at an inbred level but also at a hybrid level. Differences

in quantitative characters at a hybrid level also indicate that somaclonal variation can influence even such a complicated character as the combining ability of regenerates.

3. Improvement of regeneration of different genotypes of maize

In the first stage of this work 16 inbreds and one hybrid of different geografic origin were chosen to screen genotypes with good ability to formation of embryonic callus and regeneration. Procedure to induce callus growth from immature embryos was the same as used NOVÁK et al /1983/.

Calli produced from immature embryos were compact, nodular with visible somatic embryos after one week in culture. This imitial embryogenic callus is reffered to as Type 1 /TOMES, 1985/. After subculture on medium with 2,5 uM 2,4 D several cultures derived from inbreds CHI 31 and S 615 produced friable, light yellow calli which retained their embryogenic capacity. This type was previously designed as Type 2 /GREEN, RHODES, 1982/.

Consistent genotypic differences were observed among 16 inbreds and one hybrid in two parameters of somatic embryogenesis, i.e. in the frequency of embryogenic callus formation and the number of regenerants per cultured explant /Table 3/.

The frequency of embryonic callus formation ranged from 0 to 91,4 %. The average number of regenerated plants per explant did not reach 1% except inbreds Ms 141, S 615 and CHI 31. The latter one highly exceeded a mean value of number of regenerants per explants /14,3 %/.

Two parameters of somatic embryogenesis were generally not correlated. For example, inbreds W 37A, A 619 and hybrid Dz 7 x HMv 404 expressed a high frequency of embryonic callus formation with low or zero

plant regeneration while the inbred CHI 31 produced only 45 % embryonic callus from the plated zygotic embryos with 14,3 % of regenerated plants per explant.

Five inbreds were chosen from tested set of genotypes to back-cross with the inbred CHI 31 as a donor of somatic embryogenesis. Table 4 demonstrates the change in frequency of embryonic callus formation after 3 back-crosses. The embryonic capacity was dramatically increased in the inbreds S 7 and CM 105 /59,1 and 67,7 % respectively/ while the inbred S 615 and HMv 404 exerted 6 and 15 percent increase. The inbred W 37 A kept the same levels of embryonic capacity in back-crosses as the original non-back-crossed inbred.

If the embryonic callus formation is considered as one parameter of somatic embryogenesis the results of back-cross indicate that the somatic embryogenesis can be considered as a genetically fixed trait and can be manipulated by clasical breeding methods and introduced to the other maize genotypes.

To better know the genetic nature of the somatic embryogenesis the inbreds CHI 31 and Multiple 76-1819-3, exhibiting different embryogenic callus formation and plant regeneration abilities were reciprocally crossed /Fig. 9/. When the high embryonic inbred CHI 31 was used as a mother, the F_1 hybrid was nearly as efficient in embryogenesis and plant regeneration as for inbred CHI 31 itself. However, when CHI 31 was used as a male component in crossing, the frequency of embryonic callus formation was similar as the one in the reciprocal combination, while the number of regenerants was significantly reduced /Fig 10/. This strong maternal influence on embryonic capacity in hybrids was also evident after reciprocal back-crossing /Fig 11/ and indicate an extranuclear heritability of the somatic embryogenesis.

Conclusions

From the carried out experiments can be drawn following conclusions

1. Comparison of somaclonal variation which exists in regenerated plants from *in vitro* cultures and radiation induced variation exerted a similar spectrum of chlorophyll and morphological deviants. However, the frequency of deviants obtained in somaclones was significantly exceeding the frequency of mutagenic treated genotypes. Moreover, somaclonal variation includes much more quantitative traits such as early flowering, height of plants, compononts of yield and yield itself. The combination of explant irradiation and *in vitro* regeneration was most effective for increasing genetic variability. Regeneration ability of irradiated explants is decreased.

2. Somaclonal variability of homozygous genotypes /inbreds/ in quantitative traits can be manifested in heterozygous level /hybrids/. Results from the test of top-cross hybrids indicate changes in combining ability of regenerants and increasing genetic variability in economically important traits /yield, plant height etc./ This fact gives excellent opportunity to use the somaclonal variation in maize breeding to increase genetic variability in quantitative characters.

3. There are genotypic differences among genotypes in inducing embryogenic callus from immature embryos and regeneration ability. Results from back-crosses and test-crosses indicate that somatic embryogenesis is genetically controlled by dominant gene/s/ and genetic factors locatedin cytoplasm. As somatic embryogenesis is a genetically controlled trait, it may be manipulated with, and introduced to other genotypes by classical breeding methods /back_cross, recurrent selection etc./. This fact allowes to use the *in vitro* technique and regenerants in maize breeding programmes.

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Results on work done under the contract were published in following journals and proceedings:

NEŠTICKÝ, M., HERICHOVÁ,A., NOVÁK, F.J., DOLEŽELOVÁ, M. /1987/: Somaclonal variation in plants derived from somatic embryos of maize /Zea mays L./. Scientia Agriculturae Bohemoslovaca, 19, No.4, 253-259.

NOVÁK, F.J., HERMELIN, T., DASKALOV, S., NEŠTICKÝ, M. /1986a/: In vitro mutagenesis in maize. In: HORN, W., JENSEN, C.J., ODENBACH, W., SCHIEDER, O. /Eds./: Genetic Manipulation in Plant Breeding, pp. 563-576, Walter de Gruyter, Berlin, New York.

NOVÁK, F.J., AFZA, R., DASKALOV, S., HERMELIN, T., LUCRETTI, T. /1986b/: Assessment of somaclonal and radiation-induced variability in maize. In: Nuclear Techniques and In vitro Culture for Plant Improvement, pp. 29-33, Proc.Ser., IAEA, Vienna.

NOVÁK, F.J. , DASKALOV, S., BRUNNER, H. NEŠTICKÝ, M. AFZA, R., DOLEŽELOVÁ, M., HERICHOVÁ, A., HERMELIN, T. : Genetic variability in maize induced by tissue culture techniques. Proceedings of the 14th Congress of the Maize and Sorghum Section of EUCARPIA Nitra. Czechoslovakia, 7-11. Sep. 1987, in press.

NOVÁK, F.J., DASKALOV, S., BRUNNER, H., NEŠTICKÝ, M., AFZA, R., DOLEŽELOVÁ, M., HERICHOVÁ, A., HERMELIN, T. : Somatic embryogenesis in maize and comparison of genetic variability induced by gamma radiation and tissue culture techniques. Zeitschrift für Pflanzenz., in press.

Table [1]

FREQUENCY OF VARIANTS IN MUTAGEN TREATED, IN VITRO REGENERATED AND COMBINED GENERATIONS OF INBRED LINE CHI 31

Exp.variant	Total No. of plants	Chlorophyll variants /%/	Dwarf plants /%/	Plants with 2-3 stalks /%/	Other morpholog. variants mostly lethal /%/	All morpholog. variants /%/	Early flowering variants /%/
ES0	5186	0	0	0.10	0	0.10	0.01
ES1	4450	0.22	2.54	0.29	0.61	3.43	0.42
ES2	1263	0.24	1.98	0.32	0.48	2.77	0.07
ET0	1186	1.94	4.05	0.34	1.01	5.39	0.50
ET1	484	2.07	13.12	0.62	0.41	14.25	2.89
ET2	314	2.23	8.63	0.96	0.32	9.87	4.14

Table 2

AGRONOMIC CHARACTERS OF HYBRIDS FROM TOP-CROSS INVOLVING REGENERATED PLANTS FROM TISSUE CULTURE

Entry	Plant height /cm/	Ear position /cm/	Yield $t.ha^{-1}$
1	172	63^{++}	7.472
2	176	62	6.960^{+}
3	178^{++}	61	6.744^{++}
4	168	58	7.260
5	182	58^{++}	7.332
6	177	62^{++}	6.876^{++}
7	171	67	6.970^{+}
8	177	62	6.590^{++}
9	185^{++}	68	7.111
10	185^{++}	68	6.888^{++}
11	175	66	6.323^{++}
12	170	61	7.099
13	171	61^{+}	6.384^{++}
14	171	62	6.630^{++}
15	161^{++}	63	6.986^{+}
16	161^{++}	63	6.450^{++}
17	174	61^{+}	6.570^{++}
18	179^{++}	62	6.561^{++}
19	169	56	7.145
20	170	62^{+}	8.658^{++}
Check	171	66	7.351

Check - CHI 31 x Bu 8 Ro_2

\+ Significance at P = 0,05

++ Significance at P = 0,01

Table 3

FREQUENCY OF EMBRYOGENIC CALLUS AFTER 3 BACKCROSSES

Inbred line	Embryogenic callus of origin line /%/	Backcrossing BC 3	Embryogenic callus of back-crossed line /%/	Difference
S 615	91.4	S 615x/S 615 x /S 615xCHI 31)/	97.5	6.1
S 7	0	S 7 x /S 7 x (S 7 x CHI 31)/	59.1	59.1
HMv 404	33.3	HMv 404 x /HMv404x(HMv404xCHI 31)/	49.1	15.8
CM 105	0	CM 105 x /CM 105 x (CM 105 x CHI 31)/	67.7	67.7
W 37 A	67.6	W 37A x /W 37A x (W 37A x CHI 31)/	66.7	-0.9

CH 31 - Embryogenic inbred line

Table 1

TISSUE CULTURE RESPONSE OF INBRED LINES

Genotype	Embryogenic callus %	Mean number of regenerants per explant
GII-31	45.0	14.3
MULTIPLE 76-1019-3	36.6	0.4
S 615	91.4	2.3
RA 274	15.6	0
DZ7 x Hiv 404	61.3	0.1
W 401	8.6	0
C61	16.7	0
Dz 7	36.2	0.3
S 7	0	0
H Hv 404	33.3	0.02
RC 105	17.5	0.025
W 153 R	34.3	0.3
MS 141	55.0	1.3
GI 105	0	0
W 37A	67.6	0
A 619	56.7	0.2
Tva 218	25.6	0

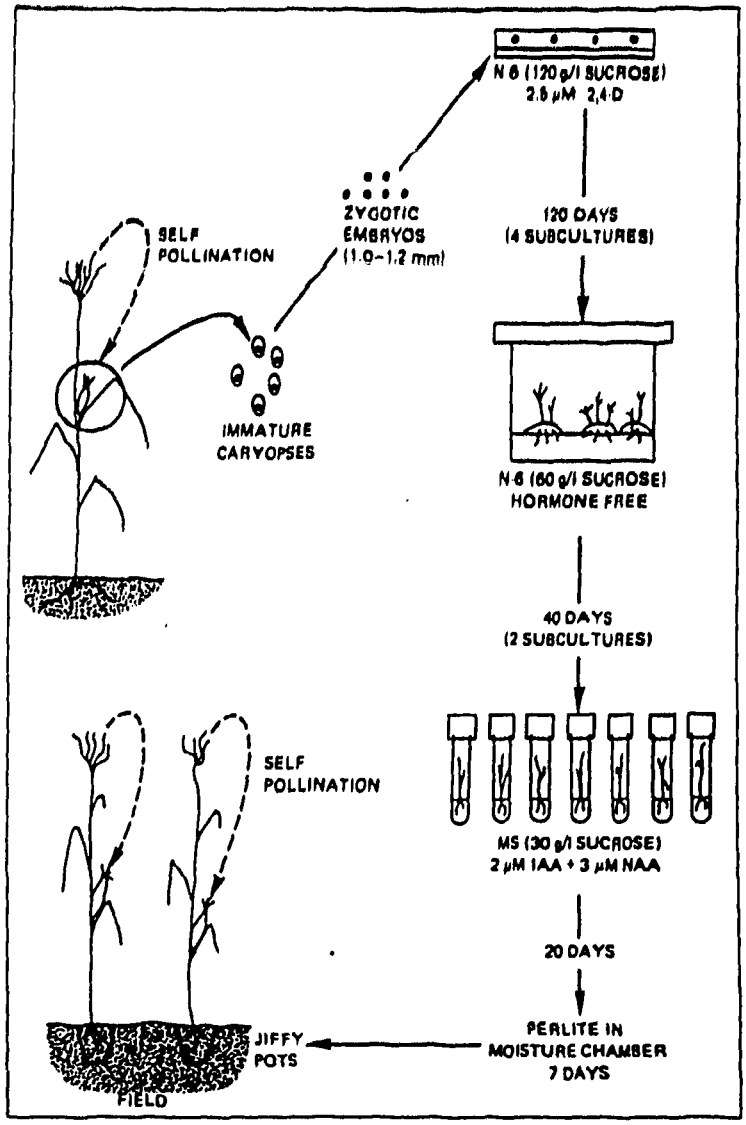


Fig. 1. Schematic representation of experimental protocol of somatic embryogenesis in maize.

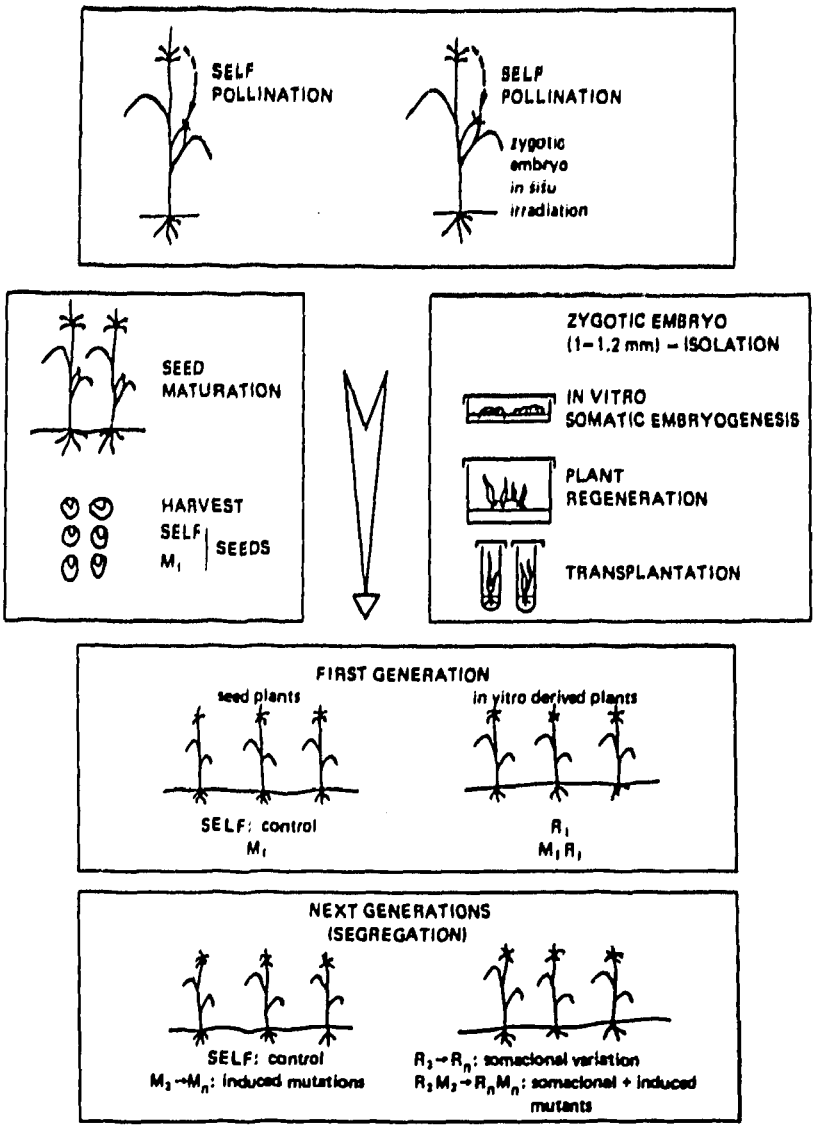


Fig. 2 Schematic representation of experiment for assessment of somaclonal and mutagen induced genetic variability.

Figure 3

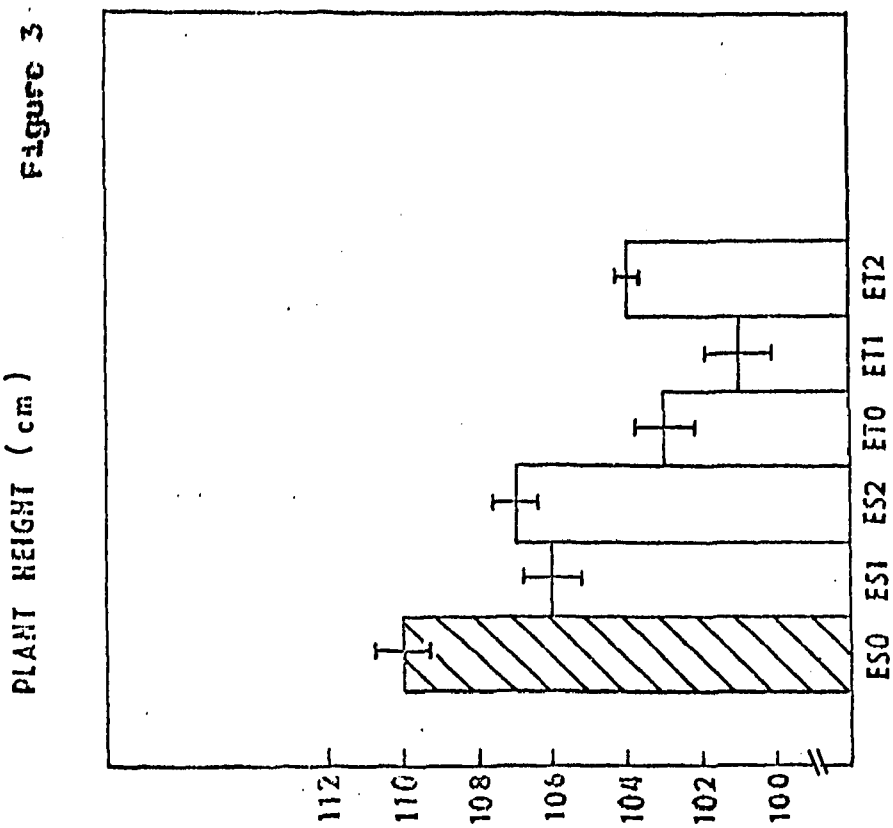
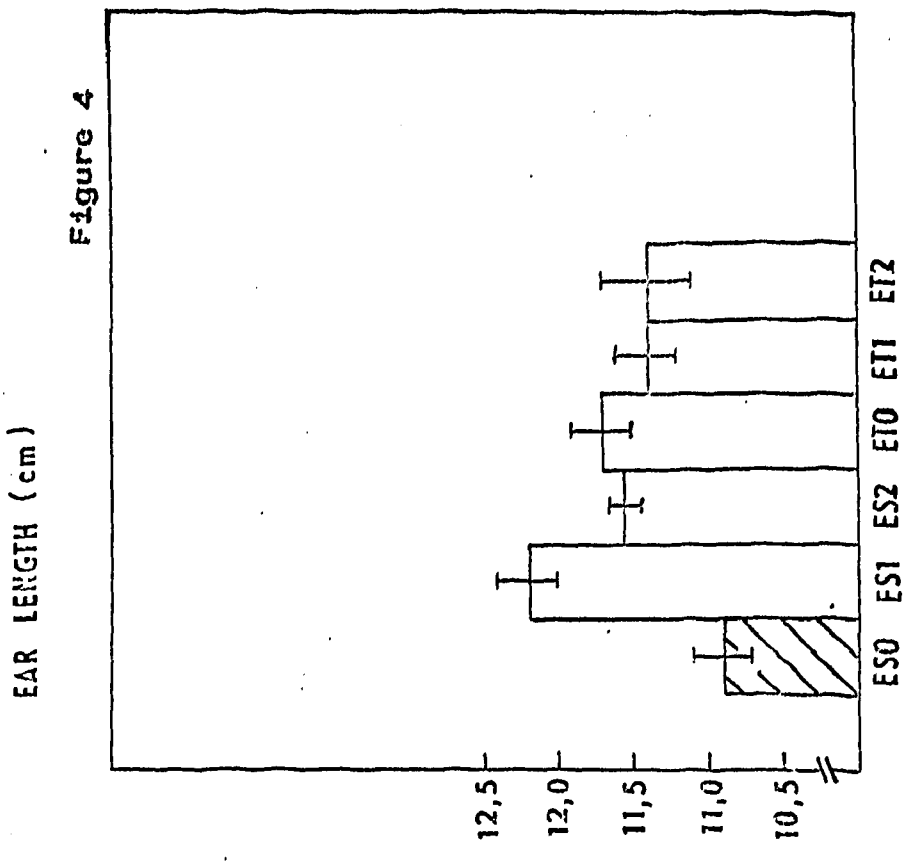

PLANT HEIGHT (cm)
112
110
108
106
104
102
100
ES0 ES1 ES2 ET0 ET1 ET2

Figure 4



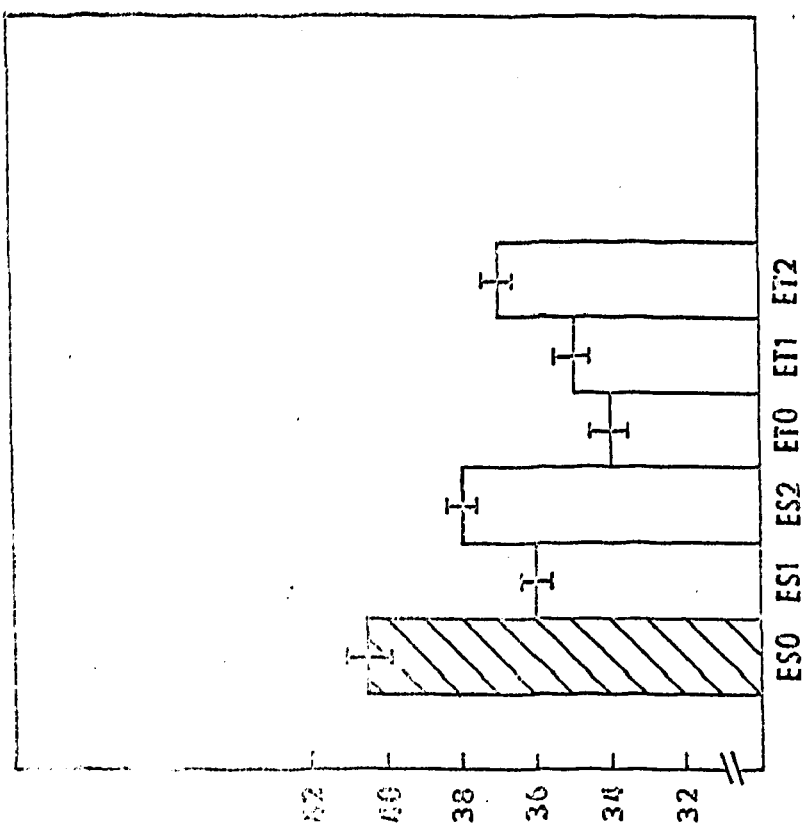
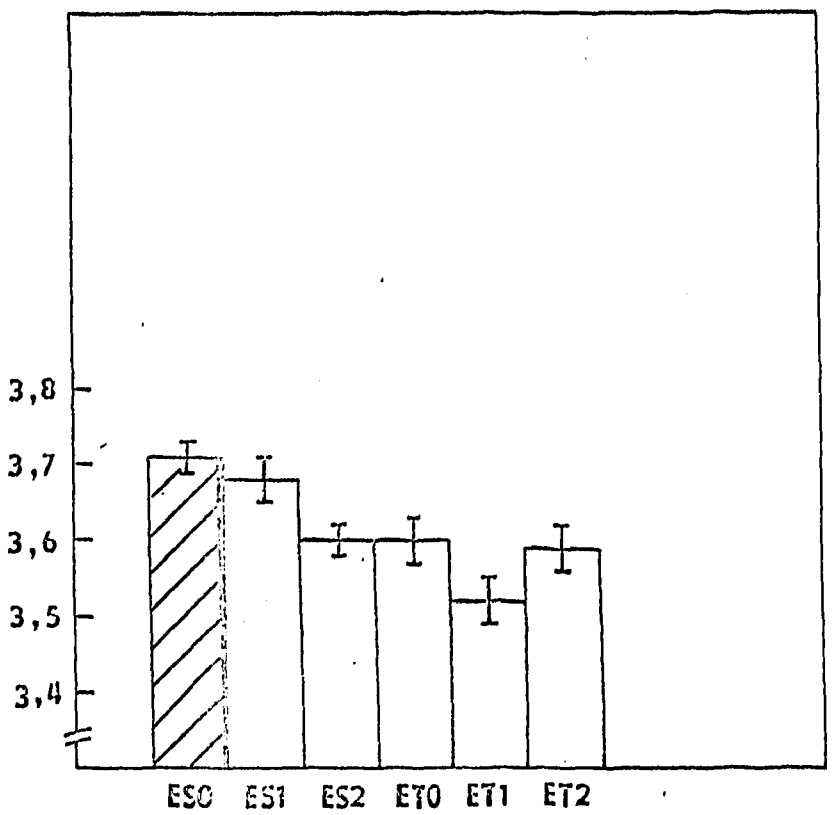


Figure 5

EAR DIAMETER (cm)

Figure 6



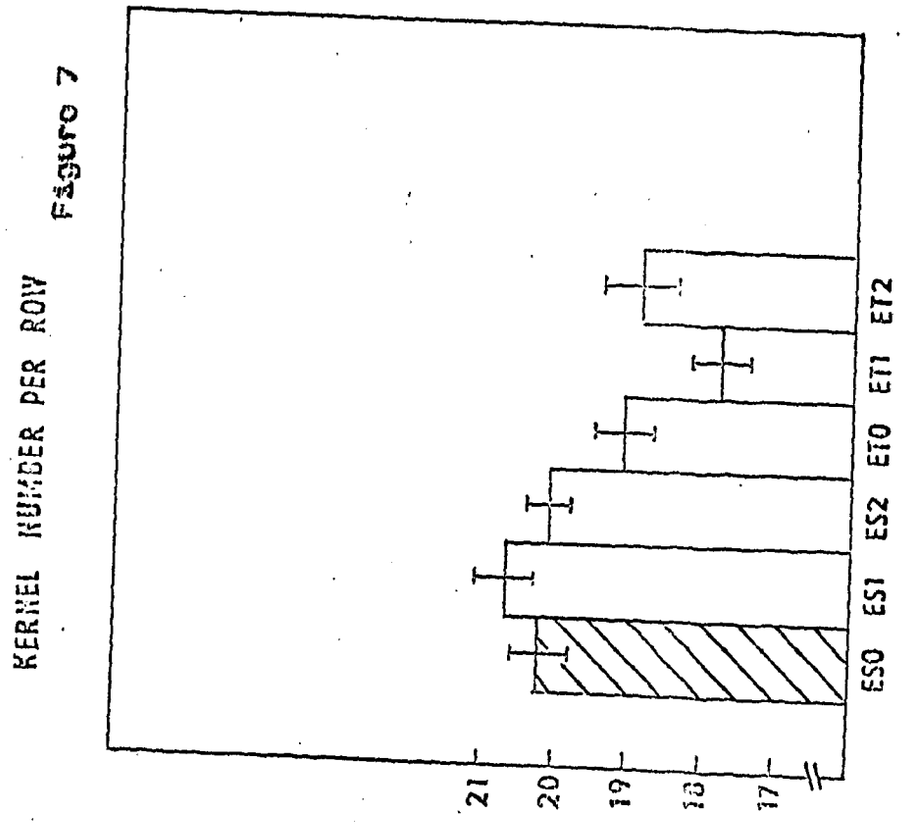
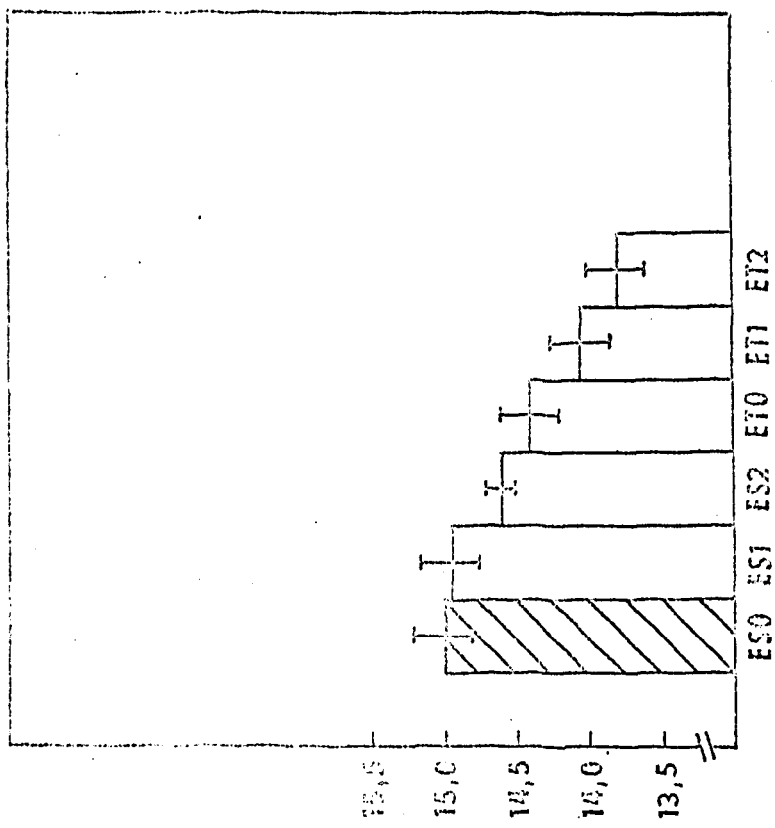


Figure 7

Figure 3



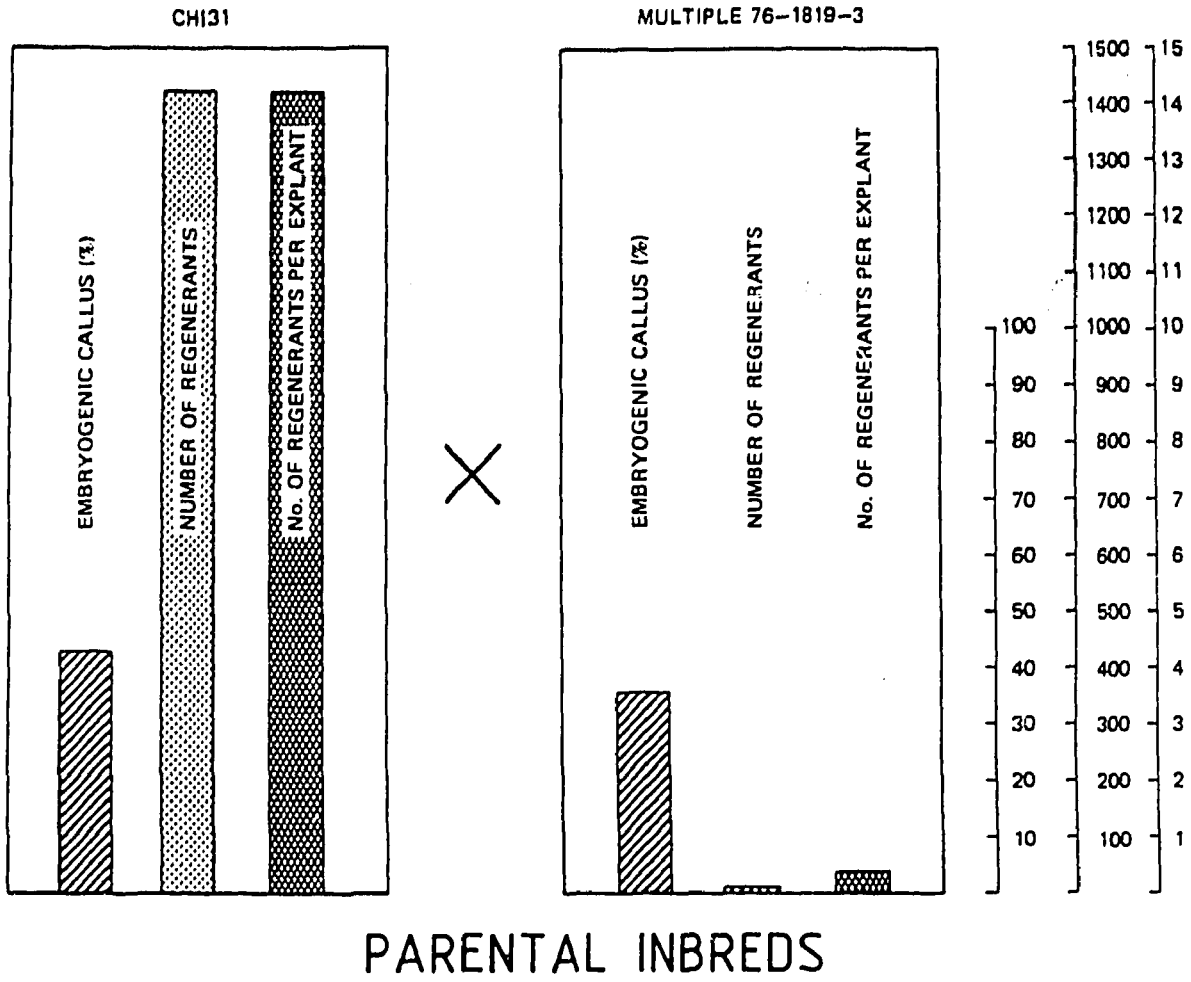


Fig. 9 Parameters of somatic embryogenesis capacity in parental inbreds.

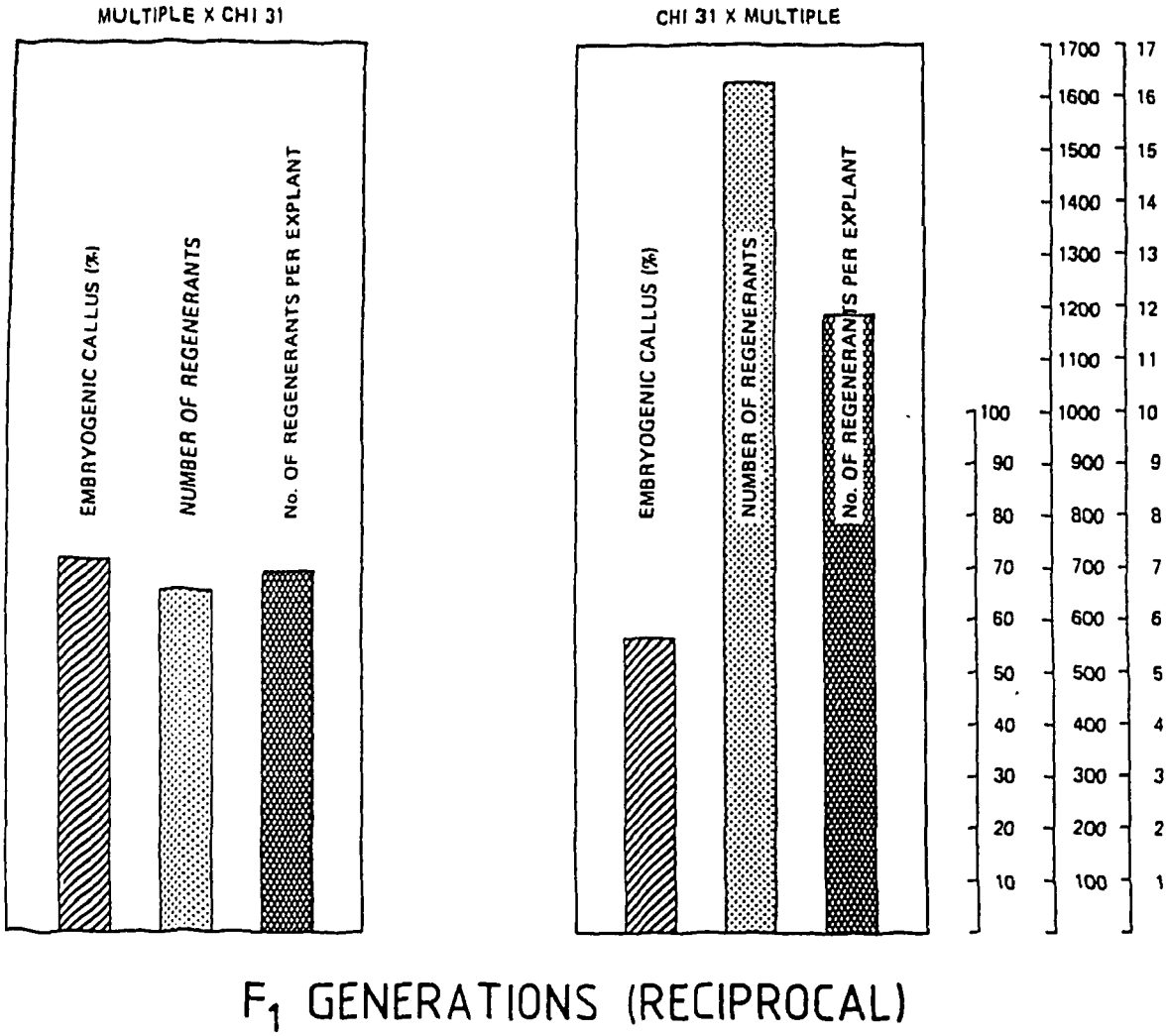


Fig. 10 Parameters of somatic embryogenesis capacity in F_1 hybrids.

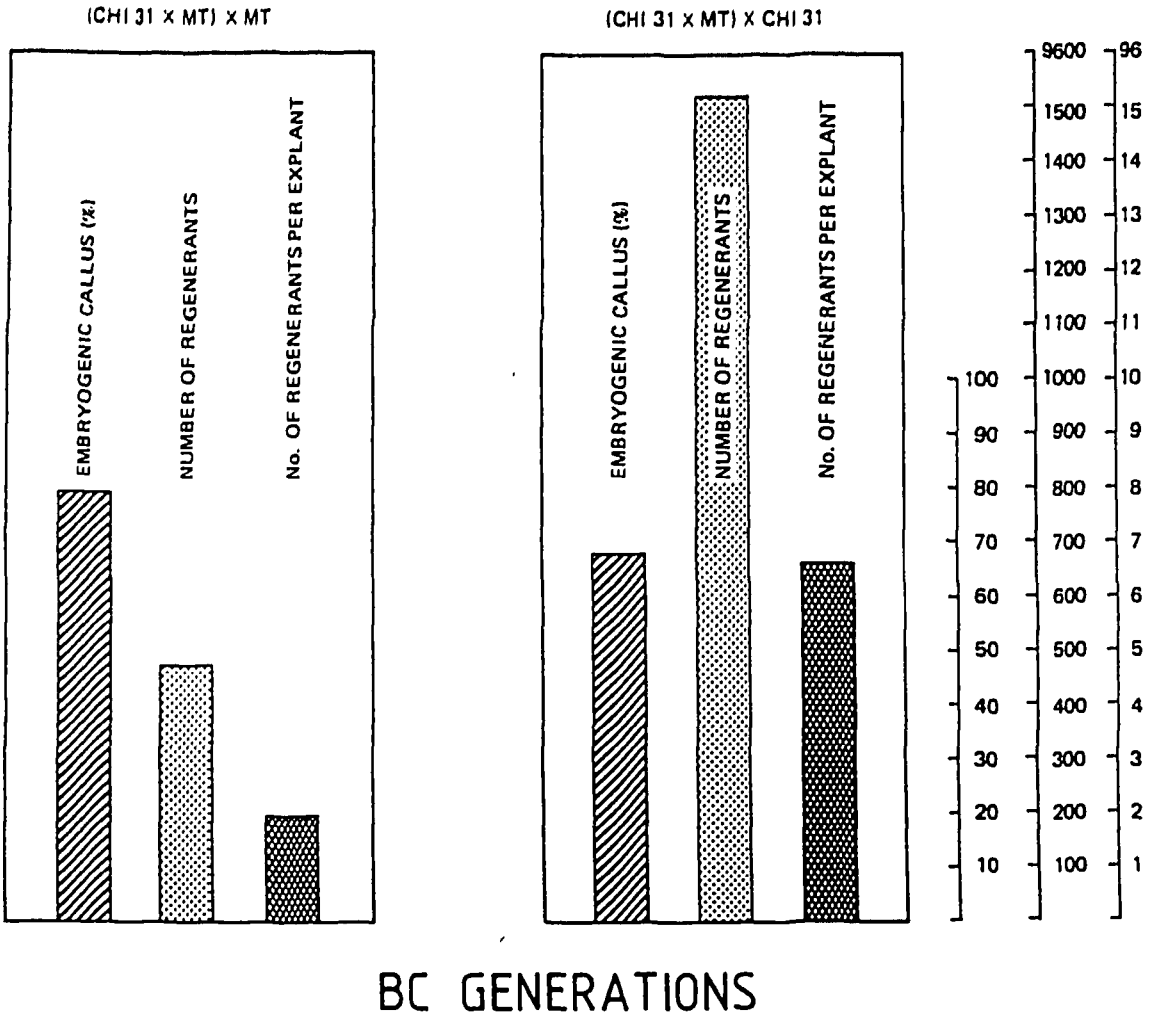


Fig.11 Parameters of somatic embryogenesis capacity in back-crosses.