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A genetic analysis of cell culture traits in tomato

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Summary. Tomato genotypes superior in regenerating plants from protoplast and callus cultures were obtained by transferring regeneration capacity from *Lycopersicon peruvianum* into *L. esculentum* by classical breeding. The genetics of regeneration and callus growth have been studied in selfed and backcross progenies of a selected plant (MsK93) which has 25% *L. peruvianum* in its ancestry. Segregation data showed that the favourable cell culture traits of *L. peruvianum* are dominant. Regeneration capacity from established callus cultures was controlled by two dominant genes. Callus growth on primary explants, callus growth of established cultures and shoot regeneration from explants had high heritabilities (0.47, 0.78, 0.87, respectively). Callus growth and regeneration capacity were not correlated within the populations studied.

Key words: *Lycopersicon* – Protoplasts – Regeneration – Callus growth

Introduction

The behaviour of plants in cell and tissue culture is determined to a large extent by medium composition and culture conditions. However, the plant species used and even the genotype within the species are also important additional factors. Intraspecific differences have often been described and analysed for particular tissue culture systems only. Examples of such genetic studies are the induction of callus on maize explants (Nesticky et al. 1983; Tomes and Smith 1985), the morphogenic response of explants in cauliflower (Buiatti et al. 1974), the response to anther culture in potato (Jacobsen and Sopory 1978) and the regeneration of shootbuds from dedifferentiated callus tissue in *Medicago* (Reisch and Bingham 1980). The interaction of genetic differences with plant hormone composition in the media has been studied in *Petunia* (Izhar and Power 1977; Skvirsky et al. 1984) and *Phaseolus* (Mok et al. 1980). For tomato (*Lycopersicon esculentum* Mill.), differences between cultivars and mutants in shoot and callus induction on primary explants have been described by many authors (Padmanabhan et al. 1974; Behki and Lesley 1976; Tal et al. 1977; Ohki et al. 1978; Frankenberger et al. 1981a; Morgan and Cocking 1982; Kurtz and Lineberger 1983; Zelcer et al. 1984). Only in two cases (Ohki et al. 1978; Frankenberger et al. 1981b) were the cultivar differences analysed by comparing hybrids with their parents. Differences between cultivars for the regeneration of shoots from subcultured callus, which in general is difficult in tomato (Locky 1983), were reported by Meredith (1979) and Tatchell and Binns (1986).

Compared to *L. esculentum,* the related species *L. peruvianum* is much easier to regenerate from long-term callus cultures without preorganized areas (Morgan and Cocking 1982; Locky 1983) and from protoplasts (Zapata et al. 1977; Mühlbach 1980; Thomas and Pratt 1981a). Also, callus growth is more abundant in *L. peruvianum.* Thomas and Pratt (1981a) suggested transferring these favourable cell culture traits from this species into *L. esculentum.* Easy regenerating tomato genotypes allow the efficient application of cell biological techniques for both basic research in a plant species that genetically is well characterized and for the genetic improvement of this important crop species. In a programme designed to obtain these genotypes (Koornneef et al. 1986), callus growth characteristics and the ability of both primary explants and subcultured callus were analysed in segregating populations derived from hybrids of *L. esculentum* and *L. peruvianum.*

Materials and methods

Plant materials

An F_3 hybrid population of *L. peruvianum* × *L. esculentum* (IVT 741505) and an *L. peruvianum* strain (PI 128650) were donated by Dr. Hogenboom of the Institute of Horticultural Plant Breeding (IVT), Wageningen, The Netherlands. The pedigree of this material is described in Koornneef et al. (1986). The F_3 population was screened for plants with the ability to regenerate shoots on callus that had been in culture for at least 1 year. Upon crossing such plants with the male sterile mutant *ms-10*^{35} of *L. esculentum* cultivar VF11, one plant (K93) gave a single offspring. This backcross hybrid plant (F_1 MsK93) was selfed to give F_2 MsK93, and was also backcrossed to VF11. The F_2 population was screened for plants that were easy to regenerate from subcultured callus and that were crossable with VF11. One F_2 plant (F_2 MsK93-19) fulfilled both criteria and from its backcross to VF11, two plants were selected for further analysis (MsK8 and MsK9). The pedigree of the material analysed genetically for cell culture traits is shown in Fig. 1.

The male sterile mutant, *ms-10*^{35} in VF11 background was a gift from Prof. Rick, Davis, USA and was maintained by seed propagation; the cultivar Bellina was a gift from the Rijk Zwaan Seed Company and the cultivar Moneymaker from Nunhems Seed Company, both in The Netherlands.

Plants of K93, MsK93, MsK8 and MsK9 were propagated by cuttings both in vitro and in vivo. Vegetative propagation was facilitated by the apparent resistance to tobacco mosaic virus of this material.

Tissue culture and protoplast techniques

Leaf discs (5 mm) were punched from surface-sterilized leaves of plants grown in a greenhouse. For shoot induction, these explants were placed on 2Z medium (Thomas and Pratt 1981b). Callus was induced on similar explants on R3B medium (Meredith 1979) with vitamins (T) as in Tewes et al. (1984). This medium was used for all callus cultures. Plant regeneration on callus was achieved by repeated transfers to 2Z medium. All cultures were grown at 25 °C and at 16 h light (approx. 2,000 lx) and transferred to new plates every 4 weeks. In all experiments 9 cm plastic Greiner Petri dishes were used.

Several cell-culture traits were assayed per individual donor plant according to the scheme of Fig. 2. Shoot-like structures were counted on the 3 best looking (out of 6) explants placed on 2Z medium for 4 weeks. At the same time, callus weight (plus original explant tissue) was determined for the three (out of six) best looking R3B explants. Six approx. 15 mg calli from the remaining pieces were transferred to new R3B plates. From these cultures, 6 calli were transferred again to R3B medium after 4 weeks; thereafter this "established" callus was used to assay the relative growth rate (RGR) by determining for the 6 calli the initial weight and that after 4 weeks. From the same source of callus material used for this RGR assay, 10 pieces were placed on 2Z medium. After 4 weeks, the best looking (green or shoot primordia containing) parts were transferred from each of these 10 calli. If after another 4 weeks on 2Z medium at least 1 of these calli showed a clearly visible shoot bud, the original plant was said to be regenerable from an established callus culture.

For protoplast isolation, plants were grown sterile in glass containers with Murashige Skoog (MS) salts, vitamins as in Tewes et al. (1984) and 1% sucrose. The plants were kept in the dark 1 day prior to harvesting of leaflets. These leaflets were floated in the dark at 4 °C on a preincubation medium: ½ strength MS salts, T vitamins, 0.5 mg l^{-1} benzylaminopurine (BA) and 1 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). The leaflets were subsequently incubated for 16 h in an enzyme solution: 0.6% Cellulase RS and 0.2% Macerozym dissolved in CPW salts (Zapata 1981), and 73 gl^{-1} mannitol.

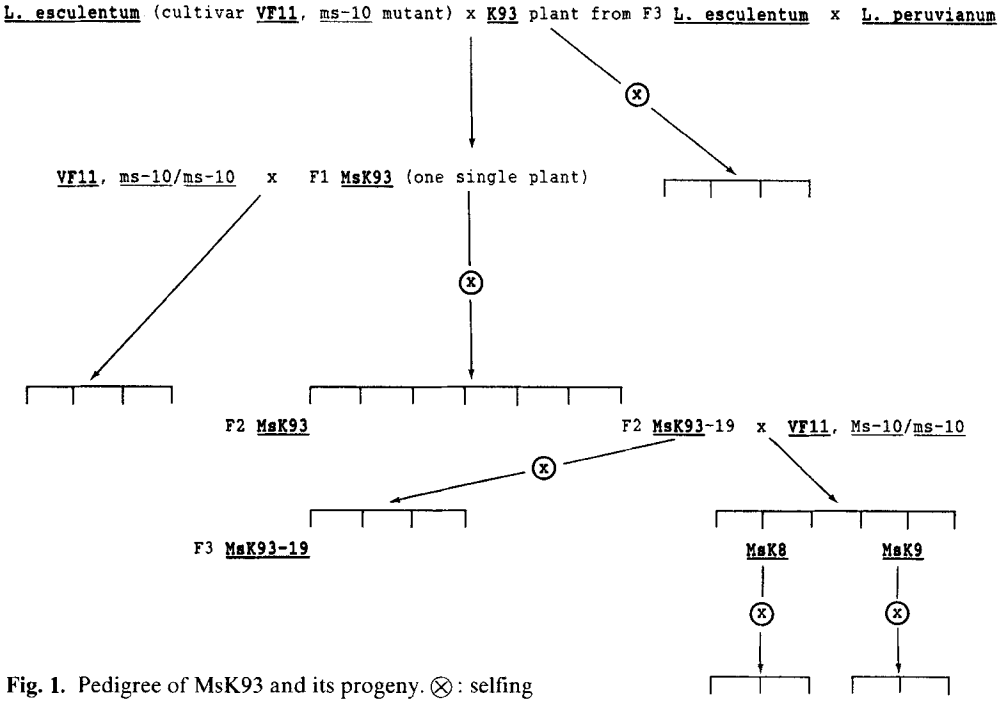

Fig. 1. Pedigree of MsK93 and its progeny. ⊗: selfing

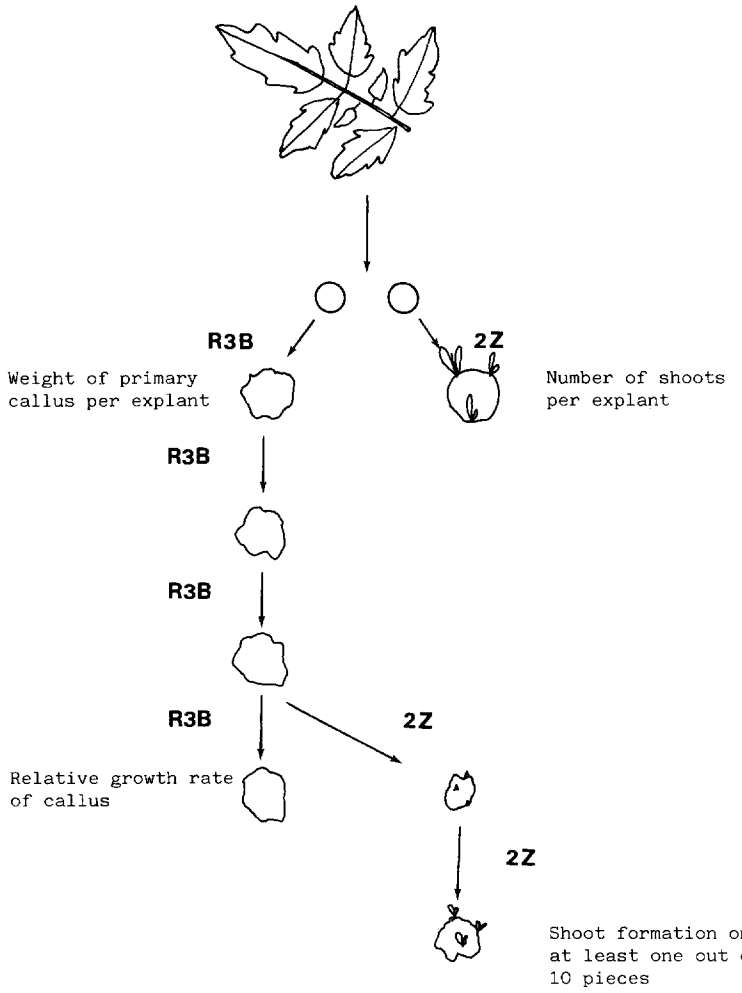

Fig. 2. Assay for cell-culture traits in tomato. *Arrows* with R3B or 2Z refer to transfer to R3B and 2Z mediums, respectively at 4 weeks intervals

The protoplasts were purified by flotation on a sucrose solution (180 gl^{-1}), using low speed centrifugation. The floating protoplasts were then pelleted in W5 wash solution (Menczel et al. 1982) and cultured in 5 ml TM-2 medium (Shahin 1985) with a higher sucrose concentration (102.7 gl^{-1} sucrose). During the liquid culture, 0.5 ml fresh modified TM-2 medium was added every 3–5 days. Sucrose (68.5 gl^{-1}), NAA (0.5 mgl^{-1}) and zeatin (0.25 mgl^{-1}) concentrations are different from Shahin's TM-2.

After 3 weeks, microcalli were transferred to a solid medium similar to Shahin's TM-3 medium, but with 36.4 gl^{-1} mannitol, 2.5 gl^{-1} sucrose, 0.5 mgl^{-1} BA and 0.1 mgl^{-1} NAA. Callus pieces from this medium were transferred to 2Z medium from which the most healthy and greenish pieces were transferred to new 2Z plates every 4 weeks until well developed shoots were present. Shoots were rooted on R3B medium with 10 gl^{-1} sucrose without hormones.

Results

Repeatability of the tissue culture systems

In the successive stages of breeding tomato genotypes with favourable cell culture traits, a number of plants of VF11, MsK93, K93, MsK8 and MsK9 were tested in replicates in different seasons (see Fig. 2 for testing scheme).

The averages of the data obtained are presented in Fig. 3. Analyses of variances for VF11 and MsK93, which were tested in all four experiments, indicated that the differences between these two genotypes and between the seasons are significant ($P < 0.01$) for the three parameters. The genotype/season interaction was not significant. The relatively low growth rate of callus in the 1984 season can be explained by the larger initial weight of the callus pieces (6 pieces of approx. 100 mg each instead of approx. 15 mg in the other seasons). For each genotype tested in replicates and in different seasons, shoot regeneration from established callus cultures (defined as described earlier) is highly reproducible (Table 1).

Genetics of cell culture traits in the progeny of MsK93

From the F_2 MsK93, obtained by selfing plant F_1 MsK93, 135 randomly chosen plants were tested for cell

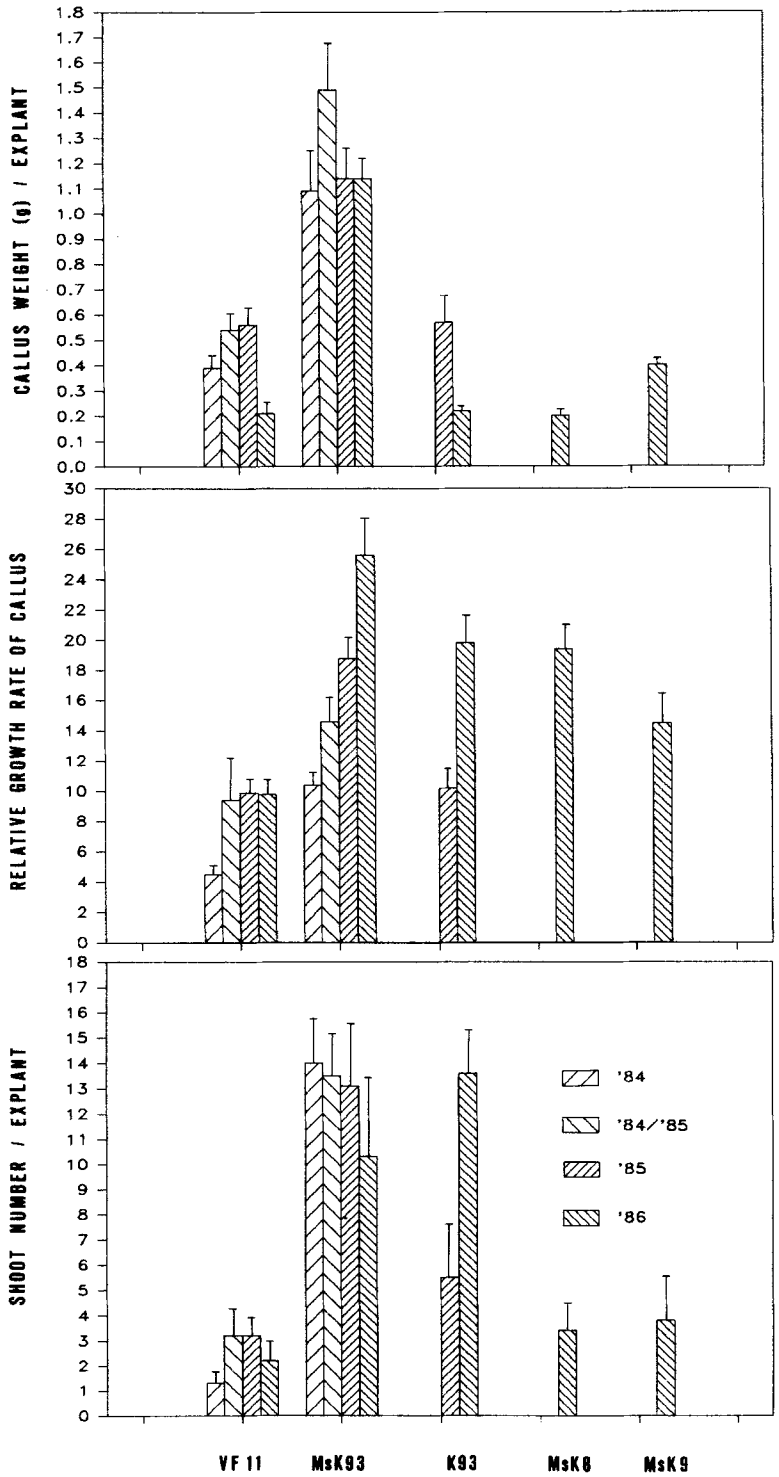

Fig. 3. Averages of plant values for weight of primary callus, relative growth rate of callus and shoot number per explant. VF11 used as seedlings, the other 4 genotypes as cuttings

culture traits in 1984 together with several VF11 and F_1 MsK93 plants. The frequency distributions are shown in Fig. 4. Shoot regeneration on primary explants is presented as ln (2+shoot number/explant) as this transformation resulted in approximately normal distributions for genetically identical plants. This was also the case for other seasons (data not shown).

Broad sense heritabilities and genetic correlation coefficients for three cell culture parameters are given in Table 2. The genetic variances and covariances required were obtained indirectly, i.e. by subtracting environmental (co) variances from total (co) variances of F_2 MsK93. The former are estimated by the total (co) variances of the isogenics VF11 and MsK93.

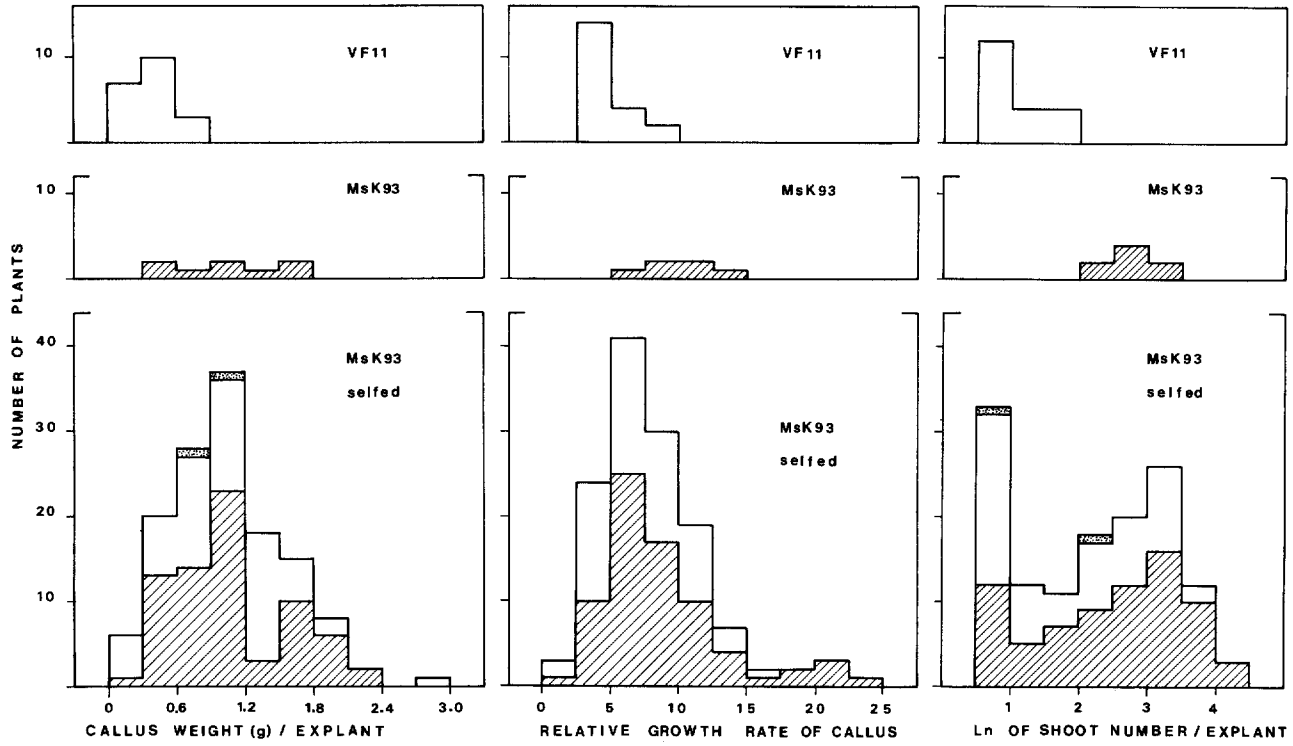

Fig. 4. Frequency distribution of plants for weight of primary callus, relative growth rate and ln (2+number of shoots/explant). *Hatched areas* indicate the number of plants that regenerate from established callus cultures. *White areas:* plants that do not regenerate from these cultures. *Dotted areas:* plants not tested for regeneration

Table 1. The number of regenerable (*R*) and non-regenerable (*N-R*) plants of genotypes that were tested in replicates and in different seasons

Season	Genotype									
	VF11		MsK93		K93		MsK8		MsK9	
	R	N-R	R	N-R	R	N-R	R	N-R	R	N-R
1984	0	20	6	0						
1984/85	0	8	5	0						
1985	0	9	10	0	9	0	1	0	1	0
1986	0	7	7	0	7	0	7	0	6	1
Totals	0	44	28	0	16	0	8	0	7	1

The heritabilities were relatively high for all traits, whereas a significant genetic correlation was only observed for the two callus growth parameters and not for shoot regeneration and callus growth. By comparing the numbers of regenerators versus non-regenerators from established callus cultures for the three parameter classes (Fig. 4) a significant positive association (X^2-test; $P < 0.01$) was observed only with shoot regeneration from primary explants. However, this association is far from absolute.

Table 2. Heritabilities (*italics*) and genetic correlation coefficients of three cell culture traits in the F_2 MsK93

	Callus weight per explant	Relative growth rate	Shoot no. per explant
Callus weight/explant	*0.47*	0.53	−0.04
Relative growth rate of callus		*0.78*	−0.03
Shoot no./explant			*0.87*

From 10 F_2 plants with the capacity to regenerate shoots from established callus cultures, F_3 progenies (in total 56 plants) were tested in a similar way to the F_2. In view of the absence of a correlation between callus growth and shoot regeneration, these 10 F_2 plants can be considered a random sample for callus growth. The correlation coefficient between these individual F_2 plants and their F_3 progeny (weighed by progeny size) was 0.27 for weight of primary callus and 0.77 for the relative growth of established callus cultures. This confirms the higher heritability for the latter trait, which was also found for the F_2 population (Table 2). Also, within the F_3 there was no significant correlation between callus growth and shoot regeneration.

Table 3. Segregation ratios for the regeneration of shoots from established callus cultures in progenies derived from K93. *R:* regenerable plants; *NR*: non-regenerable plants

Genotype	[In gene symbols]	No. of plants		Exp. ratio	χ^2 value
		R	N-R		
K93-selfed	[*Rg1*/*rg1*, *Rg2*/*rg2*⊗]	9	11	9:7	1.03
F1MsK93-selfed	[*Rg1*/*rg1*, *Rg2*/*rg2*⊗]	74	59	9:7	0.02
MsK8-selfed	[*Rg1*/*rg1*, *Rg2*/*rg2*⊗]	10	7	9:7	0.05
MsK9-selfed	[*Rg1*/*rg1*, *Rg2*/*rg2*⊗]	14	11	9:7	0.0
		107	88	9:7	0.15
L.esc.[a] × MsK93	[*rg1*/*rg1*, *rg2*/*rg2* × *Rg1*/*rg1*, *Rg2*/*rg2*]	2	18	1:3	2.41
MsK8 × *L.esc.*[b]	[*Rg1*/*rg1*, *Rg2*/*rg2* × *rg1*/*rg1*, *rg2*/*rg2*]	2	4	1:3	0.22
MsK9 × *L.esc.*[b]	[*Rg1*/*rg1*, *Rg2*/*rg2* × *rg1*/*rg1*, *rg2*/*rg2*]	3	5	1:3	0.67
		7	27	1:3	0.35
F2MsK93-19-selfed	[*Rg1*/*Rg1*, *Rg2*/*rg2*⊗]	13	2	3:1	1.1
F2MsK93-19 × *L.esc.*[a]	[*Rg1*/*Rg1*, *Rg2*/*rg2* × *rg1*/*rg1*, *rg2*/*rg2*]	4	4	1:1	0.0

[a] Cultivar VF11
[b] Cultivar Bellina

The genetics of shoot regeneration from established callus cultures

When treated as a qualitative trait, shoot regeneration, as defined before, segregates as two Mendelian genes in the progeny of MsK93 (Table 3). The differences between VF11 and plant K93 for this character are determined by two genes. K93, which in itself is heterozygous, provided the two dominant alleles responsible for shoot-regeneration from established callus culture. Apparently the F_2 plant MsK93-19 was homozygous dominant at one of the two loci. Most probably, in addition to these two major genes, minor genes also segregated in the populations studied. An indication for this is the more abundant formation of shoots on MsK93 compared to MsK8 and MsK9 when both were tested in replicates and in the same season.

Regeneration from protoplasts

Protoplast culture and shoot regeneration were compared in VF11, F_1 MsK93, MsK8 and MsK9 and *L. peruvianum* (tested under the same conditions). Fig. 5 presents the time after protoplast isolation when shoots were visible on protoplast-derived calli. These data and those in Table 4 show that regeneration of plants from protoplasts is highly efficient, especially for MsK93 and MsK9 and equals that of *L. peruvianum.* Plants regenerated from protoplasts in general look normal. However, only about one third are diploid ($2n = 24$) whereas most of the others are tetraploid ($2n = 48$). (Data to be published elsewhere.)

Table 4. Regenerations and survival of calli derived from protoplasts

Genotype	Percentage of protoplast derived calli			Total no. tested
	with shoots	without shoots	dead	
VF11	0	73	27	178
L. peruvianum	100	0	0	197
MsK93	98	0	2	350
MsK8[a]	73	3	24	115
MsK9	97	3	0	362

[a] For MsK8, data are from 150 days after protoplast isolation; data of other genotypes are from day 100 after protoplast isolation

Table 5. Mean number of germinatable seeds per fruit obtained after selfing and crossing different genotypes

Seeds obtained after	Genotype			
	MsK93	MsK8	MsK9	MM[a]
Spontaneous selfing	21.4 (10)[b]	70.6 (9)	49.2 (7)	91.4 (7)
Crosses with MM as male parent	4.8 (9)	50.2 (7)	73.2 (5)	56.8 (20)
Crosses with MM as female parent	3.2 (9)	17.3 (8)	15.3 (14)	

[a] MM: *L. esculentum* cultivar Moneymaker
[b] No. of fruits is in parenthesis

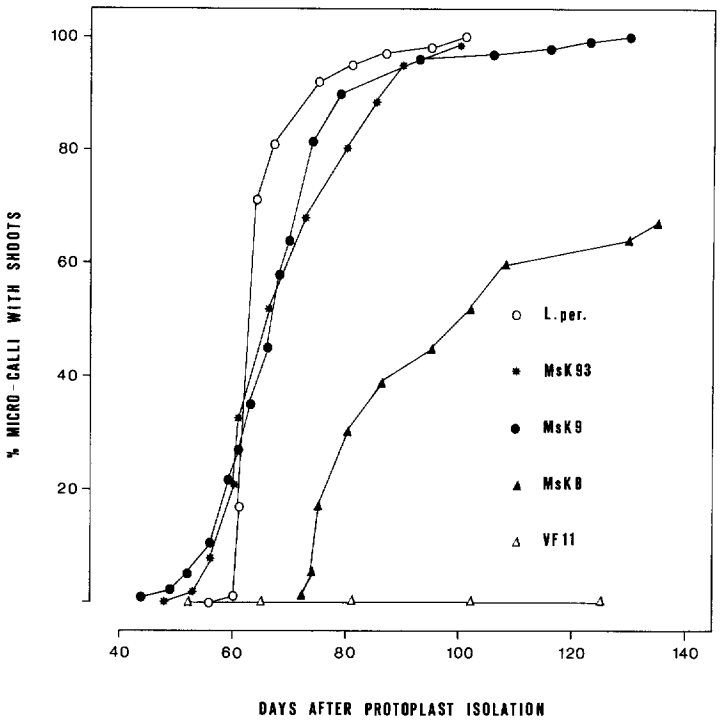

Fig. 5. Cumulative percentage of micro-calli, each derived from a single protoplast, which had produced visible shoot-like structures

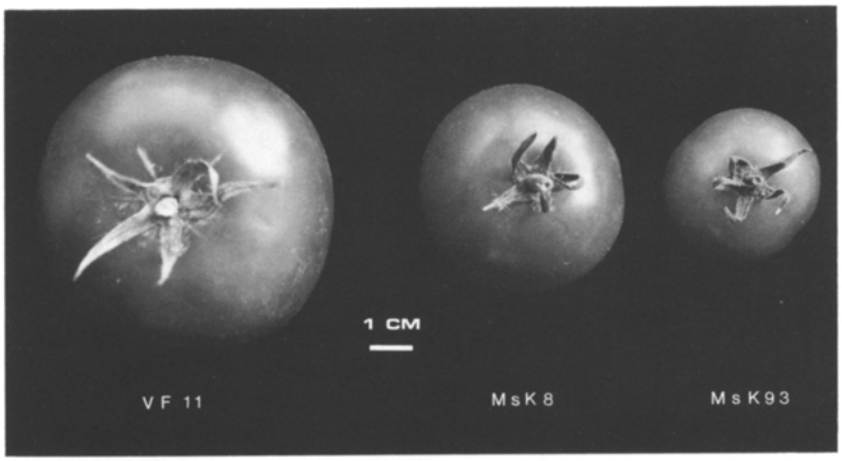

Fig. 6. Representative ripe fruits of VF11, MsK8 and MsK93

Phenotype of selected genotypes and their crossing potential with L. esculentum

When using the selected genotypes (MsK93, MsK8, MsK9) in genetic and breeding programmes of tomato a good crossing potential with other *L. esculentum* genotypes is required for easy gene transfer by normal hybridization. As can be seen from Table 5, MsK93 can be crossed with *L. esculentum* only at a low efficiency; many aborted embryo's are observed. MsK8 and MsK9 can, as female parents, be hybridized with *L. esculentum* almost as easily as cultivars of *L. esculentum* among each other. All three genotypes show a good fertility upon selfing. The most characteristic differences between *L. peruvianum* and *L. esculentum* cultivars like VF11 are their fruit size (1 cm versus 8 cm diameter) and ripe fruit colour (green versus red). Figure 6 shows how fruit sizes have increased upon repeated backcrossing with VF11. The colour of ripe fruits of MsK93 is orange/red whereas MsK8 and MsK9 have a red colour similar to VF11.

Discussion

The data presented in this paper demonstrate that the favourable cell-culture traits of *L. peruvianum* can be bred into the related species *L. esculentum*. Breeding programmes aimed specifically at obtaining genotypes

with favourable regeneration characteristics have been described for e.g. *Medicaga* (Bingham et al. 1975) and *Trifolium* (Bhojwani et al. 1984). The two programmes used genetic, within – species variation for selection. The use of germplasm from related species has been suggested, e.g. for maize where teosinte has favourable tissue culture traits (Dhaliwal and Lörz 1979; Sondahl et al. 1984).

The utility of obtaining easily regenerable genotypes in tomato is determined by the cell culture system required for a given application and by difficulties in this system for tomato. Although plant regeneration from protoplasts and established callus cultures has been reported as very difficult by several authors (as cited earlier), considerable progress has been reported recently (Shahin 1985; Niedz et al. 1985; Tatchell and Binns 1986) implying that tomato cells are also totipotent. Apparently the protocols described by these authors are sensitive to slight modifications as, for example, in our laboratory we had no success with rather similar procedures which we tested on several tomato genotypes that could be regenerated by others (data not shown).

The genotypes described in this report are especially useful when very efficient regeneration is required, which is the case when relatively rare events are selected for (e.g. somatic hybrids, rare mutants, transformants). The usefulness of MsK93 has been demonstrated in both direct gene transfer of plasmid DNA to protoplasts and in transformation experiments with *Agrobacterium tumefaciens* (Koornneef et al. 1986). As shoot regeneration from explants is relatively simple in *L. esculentum* the leaf disc *Agrobacterium* transformation procedure can also be used for tomato cultivars (McCormick et al. 1986). For transformation with the latter vector, selected tomato genotypes only increase experimental efficiency.

Dominance of the good regeneration capacity of *L. peruvianum* has been described before (Thomas 1982). Adams and Quiros (1985) used it to select somatic hybrids after protoplast fusion of *L. pennelli* (difficult to regenerate) with *L. peruvianum*. A relatively high heritability as found in this report for several cell culture traits has also been described for shoot regeneration from explants by Frankenberger et al. (1981 b), who analysed a half-diallel cross of *L. esculentum* cultivars differing in this character.

Genetic analysis of populations segregating for cell-culture traits are very scarce. In such a study on (shoot) bud regeneration from established callus-cultures of diploid alfalfa (*Medicago sativa*) Reisch and Bingham (1980) concluded that dominant alleles at two loci control good regeneration. This result is similar to what is concluded from the populations derived from the tomato genotype MsK93.

In the material studied here, no indications were found for the role of *L. peruvianum* cytoplasm in determining favourable cell culture traits of this species, because the female parent of MsK93 was *L. esculentum* with its unfavourable characteristics. Ohki et al. (1978) reported differences in organogenic capacity between reciprocal hybrids of two tomato cultivars.

The dominance of the characters tested and the simple inheritance of the traits facilitate breeding for favourable tissue culture traits in tomato. However, the selection procedure employed here is relatively laborious and it takes a long time before the regeneration potential of a plant can be classified from established callus cultures. Unfortunately, the correlation between the two callus growth characteristics (growth of primary callus and growth of established callus) is relatively poor, as is the correlation between shoot regeneration from explants and from established callus cultures. Therefore, although these early detectable traits can be used as a pre-screen, the behaviour of established cultures should be tested as well. Since callus growth and shoot regeneration apparently are different genetic traits, as was also found by Thomas (1982), selecting for both characters has to be done if both are desired.

It is difficult to speculate about the biochemical background of the genetic differences in regeneration capacity. Even a physiological description can be done in different ways. These *Rg* genes may control a quantitative difference in the number of cells that can undergo re-differentiation after de-differentiation. When this number decreases upon subculture in both *L. esculentum* and *L. peruvianum* regeneration capacity is lost much earlier in *L. esculentum*. With proper culture conditions and appropriate selection the population of organogenic cells may also be maintained for a longer period in *L. esculentum*. Alternatively, this loss of regeneration capacity may be characteristic of the *L. esculentum* alleles which cause a different epigenetic development of the cells in culture. A third explanation might be the very specific cultural conditions necessary for *L. esculentum*, whereas the requirements of *L. peruvianum* cells are far less specific. Specific interactions between hormones and genotypes have been described, e.g. in *Petunia* (Izhar and Power 1977; Skvirsky et al. 1984).

Further experiments aim at obtaining (near) isogenic lines that differ in regeneration capacity for use in further physiological analysis.

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