Theor Appl Genet (1985) 71:258–262

A study of karyotypes and their alterations in cultured and *Agrobacterium* transformed roots of *Lycopersicon peruvianum* Mill.

S. Banerjee-Chattopadhyay, A. M. Schwemmin and D. J. Schwemmin
Department of Biology, Western Washington University, Bellingham, WA 98225, USA

Received May 15, 1985; Accepted June 12, 1985
Communicated by R. Hagemann

Summary. Organ culture, plant regeneration from callus culture, and hairy root disease caused by *Agrobacterium rhizogenes* were utilized as methods of rapid in vitro propagation in *Lycopersicon peruvianum* Mill. A detailed and comparative karyotype analysis of the resulting material under such in vitro conditions revealed karyotypic stability under organ culture method, ploidy change in callus derived plants, and minor structural alterations of chromosomes in roots transformed by *A. rhizogenes.*

Key words: *Lycopersicon peruvianum* Mill. – *Agrobacterium rhizogenes* – Transformation – in vitro culture – Karyotype

Introduction

Ploidy changes and chromosomal instability in callus cultures or in plants regenerated from callus (directly or by suspension culture) have been commonly reported (Novak 1974; Liu and Chen 1976; Orton 1980; Larkin and Scowcroft 1981), whereas, organ culture has been assumed to be a method of stable, clonal propagation (David et al. 1984).

Hairy root disease of dicotyledonous plants, caused by virulent strains of *Agrobacterium rhizogenes* (Smith and Townsend 1907; Smith 1909), involves the transfer of a portion of the bacterial plasmid DNA into the host nuclear DNA and results in transformed roots which lack geotropism and produce opines (Chilton et al. 1982; Byrne et al. 1983; David et al. 1984). The possibility of using *A. rhizogenes* as a vector for the introduction of genes into higher plants (Schell and Van Montagu 1983) and the ability to regenerate shoots in vitro directly from roots (Norton and Boll 1954; Lazzeri and Dunwell 1984) has heightened interest in root organ culture.

During the course of the present investigation, in addition to organ culture, and plant regeneration from callus culture, hairy root disease caused by *A. rhizogenes* was utilized as a method for rapid in vitro propagation in *Lycopersicon peruvianum* Mill. A detailed and comparative karyotype analysis of *L. peruvianum* was undertaken to evaluate the effects of in vitro culture techniques and *A. rhizogenes* at the chromosomal level.

Abbreviations: BAP = N6-benzylaminopurine; NAA = naphthaleneacetic acid; MS = Murashige and Skoog medium; RG = regeneration medium; SDS = sodium dodecyl sulfate

Materials and methods

Lycopersicon peruvianum seeds (supplied by Glasshouse Crops Research Institute, Littlehampton, Sussex, England) were grown in soil and were maintained as control group at 27 °C with 16 h of low intensity illumination and 8 h of darkness.

The various techniques applied for in vitro propagation and cytological studies in *L. peruvianum* are as follows.

In vitro techniques

1 Roots from seeds. Seeds were surface sterilized with 20% commercial bleach (Clorox) plus 0.1% SDS for 20 min; immersed in 70% ethanol for 2 min; and then rinsed in sterile, distilled water. After germination on water agar, roots were excised and transferred to 125 ml Erlenmeyer flasks containing 25 ml of R3B liquid medium (Meredith 1979) without hormones under both static and shaker (100 rpm) conditions and on the same medium solidified with 0.9% agar in 100 × 25 ml Petri dishes. All cultures were maintained under the same temperature and photoperiod as the control group.

2 Transformed roots. L. peruvianum seeds were grown in soil under greenhouse conditions. Internodal stem segments, 2–4 cm long, were surface sterilized and embedded in 12 ml of LSS agar medium (Linsmaier and Skoog 1965) with no hor-

mones in culture vials, leaving a cut surface exposed. The exposed cut surface was inoculated with *Agrobacterium rhizogenes* $C_{58}ClpR_i15834$ (supplied by Max-Planck-Institut für Züchtungsforschung, Köln, FRG). Inoculation was directly from colonies grown for 2 days on yeast extract peptone (YEP) agar (Barton et al. 1983). Roots developed on the cut surface after 7 d and were transferred to R3B liquid medium with 100 mg/L carbenicillin and no hormones. Bacteria-free transformed roots were maintained on R3B (no hormones) liquid medium in both static and shaker conditions and on the same medium solidified with 0.9% agar. The transformation of such roots was confirmed as the preliminary electrophoresis of the root extracts indicated the presence of specific silver nitrate positive substances one of which was most likely mannopine.

3 Roots from callus. Petiole explants of *L. peruvianum* were surface sterilized and placed on R3B agar with 2 mg/L NAA and 1 mg/L BAP. Callus was apparent after 10 days and was subsequently maintained on this medium. Shoots were regenerated from callus on the RG medium of Zapata et al. (1977). Roots were induced by placing the regenerated shoots on MS+0.1 mg/L NAA (Morgan and Cocking 1982). These roots were then grown on R3B (no hormones) liquid medium in both static and shaker conditions, and on the same medium solidified with 0.9% agar.

Cytological techniques

For the study of the somatic chromosomes, root tips were collected from soil grown control plants and from those resulting from all the culture conditions mentioned above. The root tips were pretreated in half saturated paradichlorobenzene solution for 3 h at 10 °C with an initial 3 min shock treatment at 0–5 °C. Overnight fixation in an acetic acid : ethanol mixture (1 : 3) was followed by 15 min of cold hydrolysis in 5 N HCl at 10 °C (Chattopadhyay and Sharma 1983). This was followed by thorough washing in water, 5 min treatment with 45% acetic acid and subsequent overnight staining in 2% aceto-orcein. The root tips were finally squashed in 45% acetic acid following the usual technique (Sharma and Sharma 1982).

Results

The somatic chromosome number of the soil grown control plants of *L. peruvianum* was found to be 24 (Fig. 1 a). This number was also prevalent in both the roots either transformed in origin or from seeds, irrespective of the culture conditions (Fig. 1 b and c). All the roots from callus derived plants, grown under the three different culture conditions were found to be tetraploid (Fig. 1 d).

Variation in the number of chromosomes were more prevalent in roots grown in liquid than those grown in solid medium (Fig. 1 e–l). In the liquid medium, the percentage of variation was almost the same under static or shaker conditions (Table 1). However, the karyotype analysis revealed a general morphological similarity between the complements of the different materials studied and provided the basis from which six chromosome types were formulated.

Type A. Chromosome with secondary constriction whose distal part was widely separated from the rest and remained attached by a DNA positive thread, the exact nature of which was unknown. The distal segment and the median segment were almost equal in size. This was a characteristic chromosome of *L. peruvianum* found in all the materials studied.

Type B. Chromosome with two constrictions, one median and the other sub-median in position.

Type B′. Chromosome with two constrictions with the distal segment smaller than the other two arms which were equal in size.

Type C. A secondarily constricted chromosome, with an end segment larger than the other one, and the middle segment still larger than the two distal ones.

Type D. Chromosome with a nearly submedian primary constriction.

Type E. Chromosome with a nearly median to median primary constriction.

The chromosome numbers and the karyotype formulae of the different samples investigated are as

Table 1. Variations in chromosome number and percentage of variant nuclei under different in vitro conditions

Name of sample	Variation no. of chromosomes			% of variant nuclei		
	Solid medium	in liquid medium		Solid medium	in liquid medium	
		Static	Shaker		Static	Shaker
Roots from plants regenerated from callus	9; 32	12; 20	5; 14; 20; 36	2%	2%	5%
Roots from transformed plants by *A. rhizogenes*	×	11	11; 20; 23	0	1%	3%
Roots from seeds germinated under in vitro conditions	×	11; 12; 14; 16	12; 20; 22; 48;	0	4%	4%

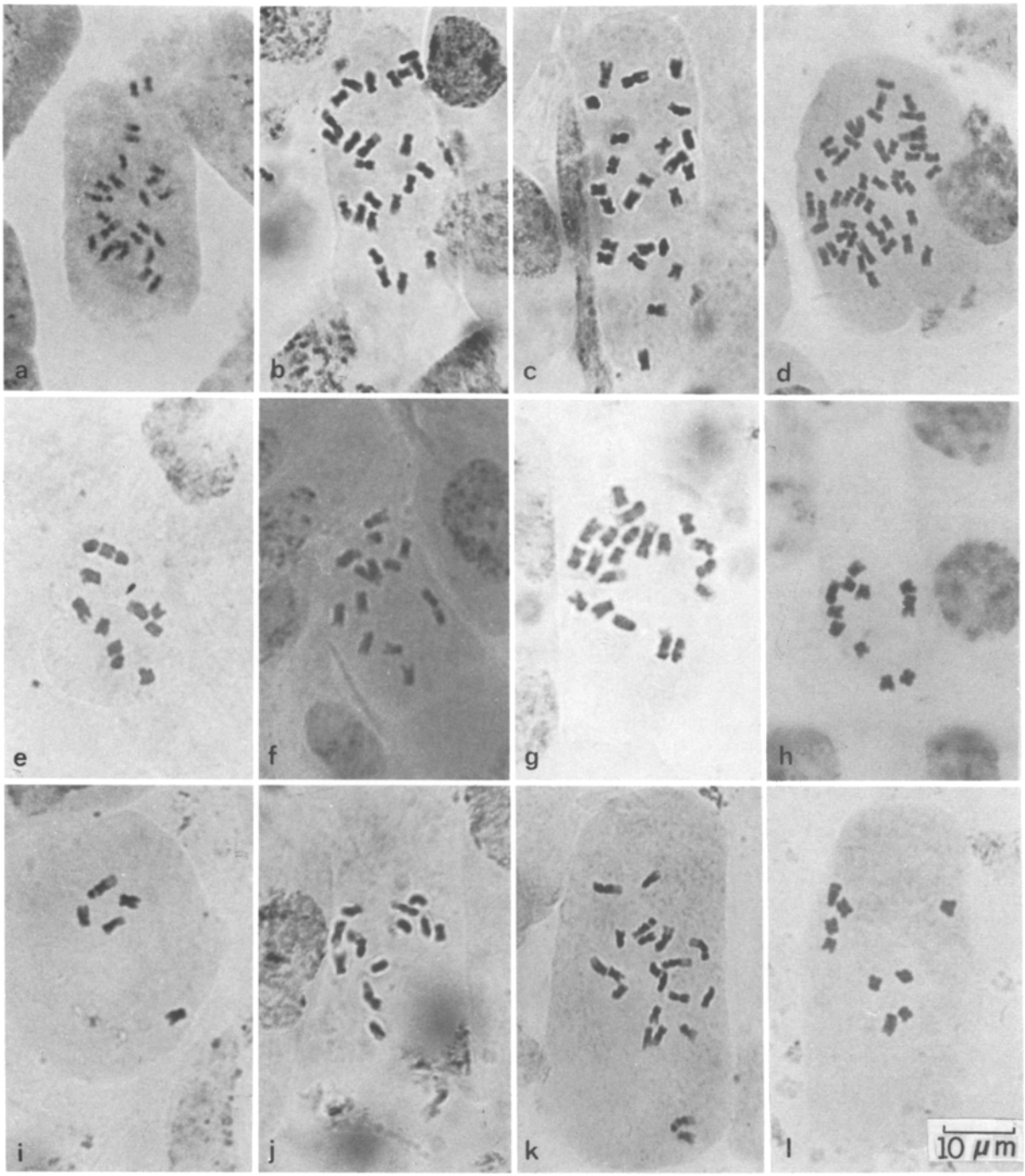

Fig. 1 a–l. Somatic metaphase nuclei of *Lycopersicon peruvianum* Mill. **a** control roots, 2n = 24; **b** transformed roots by *A. rhizogenes*, 2n = 24; **c** roots from in vitro germinated seeds, 2n = 24; **d** roots from callus derived plants with 2n = 48; **e–g** variation numbers in roots from in vitro germinated seeds showing 12 chromosomes (solid medium), 14 and 20 chromosomes (liquid medium) respectively; **h** variation number in transformed root showing 11 chromosomes (solid medium); **i–l** variation numbers in roots from callus derived plants showing 5, 12, 20 chromosomes (liquid medium) and 9 chromosomes (solid medium), respectively

follows:

Roots from soil (Control)	(Diploid)	$2n=2x=24=2A+4B+4C+10D+4E$
Roots from seed	(Diploid)	$2n=2x=24=2A+4B+4C+10D+4E$
Transformed roots	(Diploid)	$2n=2x=24=2A+2B+6C+10D+4E$
Roots from callus-derived plants	(Tetraploid)	$2n=4x=48=4A+6B+2B'+6C+20D+10E$

If we consider the basic karyotype or genome as, $X=A+2B+2C+5D+2E$, the karyotype formulae will appear to be:

Roots from soil (Control)	$2n=24=2(A+2B+2C+5D+2E)=XX$
Roots from seed	$2n=24=2(A+2B+2C+5D+2E)=XX$
Transformed roots	$2n=24=2(A+2B+2C+5D+2E)+2C-2B=XX+2C-2B$
Roots from callus-derived plants	$2n=48=4(A+2B+2C+5D+2E)+2B'+2E-2B-2C=XXXX+2B'+2E-2B-2C$

The karyotype formulae were found to be constant within each sample growing either on solid or in liquid medium and either in static or shaker condition. As such, one karyogram of each sample has been represented in Fig. 2. The chromosome size, in this investigation, ranged from 3.93 μm to 1.96 μm.

Discussion

The somatic chromosome number of *L. peruvianum* was found to be 2n=24. This was reported earlier by Luckwill (1943). Under the prevalent culture conditions, the roots from seedlings and the transformed roots represented the diploid number, i.e., 2n=24, as their modal numbers. All the callus derived plants revealed tetraploidy with 2n=48 chromosomes. Tetraploidy in callus derived tomato plant was reported earlier by Evans et al. (1984). High probability of chromosomal variation is well documented in case of callus culture but the final genotypic constitution of regenerated plants depends more on the relative competitive abilities of different genotypes than on the frequency of their origin (Bayliss 1980). Since all the callus derived plants so far studied in this species were found to be tetraploid, the selective advantage of the tetraploid cells over all other variant cells is apparent.

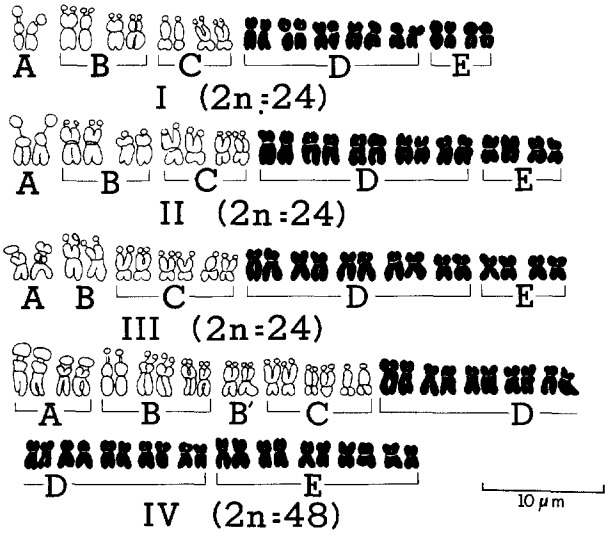

Fig. 2. Comparative karyogram of *L. peruvianum* under different culture conditions. I=Control plant; II=Roots from in vitro germinated seed; III=Transformed roots by *A. rhizogenes;* IV=Roots from callus derived plants

Roots from seedlings cultured on solid or liquid medium, displayed exactly the same karyogram as the control. This indicates that, at least in this case, the culture conditions were not inducing any karyotypic alterations. A similar result was also reported in *Tradescantia,* where tissue culture media, regardless of its hormonal composition, was found to have no direct effect on induction of mutations (Dolezel and Novak 1984).

The karyogram of transformed roots revealed two B and six C chromosomes as opposed to four B and four B in control plants. Such structural alterations might have been induced during transformation by *A. rhizogenes.* A DNA probe of Ri origin should be utilized to investigate such chromosomal alterations.

The karyogram of callus derived plants, although displaying tetraploidy, the chromosome types were not the exact multiples of the diploid set. This suggests the influence of structural alterations of chromosomes associated with polyploidy. The appearance of two new B′ type chromosomes were further indicative of the role of structural alterations which might have involved two B and two C type chromosomes and resulted into two B′ and two E types.

Besides the modal numbers of each group studied, wide range of variations in chromosome numbers were noted during the present course of investigation. Comparatively higher frequency of variant nuclei was observed amongst the liquid grown cultures than in the solid grown ones. The probable mode of origin and fate of such variant nuclei were extensively reviewed by Bayliss (1980). The liquid grown roots, either in static or shaker condition, appeared to grow more vigorously which eventually involved rapid cell division. Non-dysjunction of chromosomes and such rapid growth rate may account for the high frequency of variant nuclei in liquid grown roots.

The results reported here suggest that transformed roots (by *A. rhizogenes*) were most effective for rapid in vitro micropropagation, as, in addition to their faster growth rate, they retained the normal diploid karyotype with minor structural alterations between the complements.

Acknowledgements. Thanks are due to Dr. J. Schell, Max-Planck-Institut für Züchtungsforschung, Köln, FRG, for providing the culture of *A. rhizogenes*; and John W. Maxon Smith, Glasshouse Crops Research Institute, Littlehampton, Sussex, England, for supplying seeds of *L. peruvianum*. At the department of Biology, Western Washington University, we thank Drs. G. Kraft and M. Dube for extending the research facilities, J. Rosemary Read, for tissue culture establishment and maintenance, and Douglas Doolittle for developing a number of photographs. We are especially grateful to Eugene Hoerauf, Geography and Regional Planning, WWU, for his invaluable assistance in preparing the illustrations.

References

Barton KA, Binns AN, Matzke AJ, Chilton MD (1983) Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R_1 progeny. Cell 32:1033–1043

Bayliss MW (1980) Chromosomal variation in plant tissues in culture. In: Vasil IK (ed) Perspectives in plant cell and tissue culture. Acadmic Press, New York London, Int Rev Cytol, Suppl 11A, pp 113–144

Byrne MC, Koplow J, David C, Tempe J, Chilton MD (1983) Structure of T-DNA in roots transformed by *Agrobacterium rhizogenes*. J Mol Appl Genet 2:201–209

Chattopadhyay S, Sharma AK (1983) Genetic diversity in *Costus speciosus* (Koen.) Sm. Cytologia 48:209–214

Chilton MD, Tepfer DA, Petit A, David C, Casse-Delbart F, Tempe J (1982) *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. Nature 295: 432–434

David C, Chilton MD, Tempe J (1984) Conservation of T-DNA in plants regenerated from hairy root cultures. Bio/Tech 2:73–76

Dolezel J, Novak FJ (1984) Effect of plant tissue culture media on the frequency of somatic mutations in *Tradescantia* stamen hairs. Z Pflanzenphysiol 114:51–58

Evans DA, Sharp WR, Medina-Filho HP (1984) Somaclonal and gametoclonal variation. Am J Bot 71:759–774

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214

Lazzeri PA, Dunwell JM (1984) In vitro shoot regeneration from seedling root segments of *Brassica oleracea* and *Brassica napus* cultivars. Ann Bot 54:341–350

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Liu MC, Chen WH (1976) Tissue and cell culture as aids to sugar cane breeding. 1. Creation of genetic variation through callus culture. Euphytica 27:273–282

Luckwill LC (1943) The genus *Lycopersicon*, an historical, biological, and taxonomic survey of the wild and cultivated tomatoes. Aberdeen University Studies No 120. The University Press, Aberdeen, pp 5–44

Meredith CP (1979) Shoot development in established callus cultures of cultivated tomato (*Lycopersicon esculentum* Mill). Z Pflanzenphysiol 95:405–411

Morgan A, Cocking EC (1982) Plant regeneration from protoplasts of *Lycopersicon esculentum* Mill. Z Pflanzenphysiol 106:97–104

Norton JP, Boll WG (1954) Callus and shoot formation from tomato roots in vitro. Science 119:220–221

Novak FJ (1974) The changes of karyotype in callus cultures of *Allium sativum* L. Caryologia 27:44–54

Orton TJ (1980) Chromosome variability in tissue cultures and regenerated plants of *Hordeum*. Theor Appl Genet 56: 101–112

Schell J, Van Montagu M (1983) The T_i-plasmids as natural and practical gene vectors for plants. Bio/Tech 1:175–180

Sharma AK, Sharma A (1982) Chromosome techniques: theory and practice. Butterworth, London Boston

Smith EF (1909) The etiology of plant tumors. Science 30:223

Smith EF, Townsend CO (1907) A plant tumor of bacterial origin. Science 25:671–673

Zapata FJ, Evans PK, Power JB, Cocking EC (1977) The effect of temperature on the division of leaf protoplasts of *Lycopersicon esculentum* and *Lycopersicon peruvianum*. Plant Sci Lett 8:119–124