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Efficient Hybridization Between *Lycopersicon esculentum* and *L. peruvianum* via Embryo Callus

B.R. Thomas and D. Pratt
Department of Bacteriology, University of California, Davis, California (USA)

Summary. Embryo callus was produced in up to 12% of the undeveloped seeds of the interspecific cross *L. esculentum* cv. VFNT cherry × *L. peruvianum* LA1283-4, a cross which does not produce viable seeds. Plants were produced from 90% of those callus clones by regeneration and rooting. Evidence that these plants were hybrid includes morphology, isoenzyme patterns and fertility relations. Both diploid and tetraploid plants were produced, 40% of the regenerated callus clones producing at least one diploid plant. Thus, up to 4% of the undeveloped seeds plated for callus production eventually yielded diploid hybrid plants. In contrast, among 401 undeveloped seeds dissected, no embryos suitable for embryo culture were found. A backcross of the embryo callus hybrids to the *L. esculentum* parent has succeeded – producing, as expected, only undeveloped seeds; these undeveloped seeds have produced callus.

Key words: Interspecific hybridization – Embryo callus – Plant regeneration – *Lycopersicon esculentum* – *Lycopersicon peruvianum*

Introduction

Interspecific hybridization is an important tool for introducing valuable traits from wild species into the gene pools of cultivated plant species. A unilateral incompatibility mechanism prevents *Lycopersicon esculentum*, the cultivated tomato, from serving as the male parent in crosses with *Lycopersicon peruvianum*, a wild related species. Pollen tube elongation is normally stopped before fertilization is effected. Ovule penetration by any pollen tubes that happen to elongate sufficiently is also inhibited. The result is that no fruits are set (Hogenboom 1972b). When *L. peruvianum* is the male parent, the cross is prevented by embryo abortion. Fruits are set, but no viable seeds are formed (Cooper and Brink 1945; Hogenboom 1972a).

Each of the reciprocal crosses can succeed under certain circumstances. Hogenboom has selected rare strains of *L. peruvianum* in which the unilateral incompatibility barriers have been partially broken. Interspecific hybrids were produced from fully developed seeds with *L. esculentum* as the male parent (Hogenboom 1972b, c). Because no successful backcross of these hybrids to the *L. esculentum* parent has been reported, this method remains unproven as a way of transferring alleles from one species to the other. In the interspecific crosses where *L. peruvianum* is the male parent, plants have been successfully produced by embryo culture (Smith 1944). Embryo culture is needed again for the first backcross of the hybrid to *L. esculentum*. Subsequent backcrosses form normal seeds (Alexander 1963).

This paper describes an improved method for production of F_1 interspecific hybrids between *L. esculentum* and *L. peruvianum* that is far more efficient that conventional embryo culture and does not restrict the choice of female parents to the strains selected by Hogenboom.

The work described in this paper was begun as part of an attempt to backcross into *L. esculentum* the rapid callus production and efficient shoot regeneration traits found in *L. peruvianum*. A report of our construction of tomato strains for cell culture research will appear elsewhere.

Materials and Methods

Stocks

VFNT cherry, LA 1221, (P.G. Smith, Dept. of Veg. Crops, Univ. of Calif., Davis) is the *L. esculentum* inbred strain used in this work. Its greatest advantage is its rapid multiplication, approaching four generations per year. Under ideal conditions, VFNT will produce ripe, red fruits within six weeks after pollination. LA 1283-4 is a strain deriving from a wild population of *L. peruvanium*

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found at Sta. Cruz de Laya, Peru (C.M. Rick, personal communication). It was selected for this work because of its exceptionally good callus production and plant regeneration abilities. Both LA 1221 and LA 1283 seeds were obtained from the Tomato Genetics Cooperative, Dept. of Veg. Crops, Univ. of Calif., Davis.

Embryo Callus and Plant Regeneration

For embryo callus production fruits with unbroken skins were sterilized by first dipping them in 95% ethanol and then soaking them for five minutes in 5% sodium hypochlorite. All subsequent operations, through to plating the seeds, were conducted under sterile conditions. Over 1000 undeveloped seeds were plated without any losses due to contamination. Fruit dissection and removal of the jelly-like coating on the seeds were carried out in a petri dish on a bed of sterile absorbent paper. The media used are described in Table 1. The 10-100 undeveloped whole seeds recovered from each fruit were plated on a single 9 cm petri plate. Callus was initiated and maintained in the dark at 27°C. Shoot regeneration from callus and root production from shoots were done at room temperature (20-30°C) with 3000 lux of fluorescent light for 16 hours per day.

Embryo Culture

Embryo culture was attempted by dissecting seeds 35 days after pollination. While this is less than the 40 days after pollination used by Nettancourt et al. (1974) and at the low end of the 35-40 day range used by Smith (1944), this time was chosen because VFNT fruit development is more rapid than that of the strains used by these workers. Thirtyfive days after pollination is also within the time range during which we observed the peak efficiency of embryo callus production. Embryos were dissected out from VFNT fruits of various ages to observe different developmental stages to help insure that any embryos in the undeveloped hybrid seeds would be recognized.

Table 1. Media

Medium	Purpose	Hormone composition
2D/1P CCM[a,d]	Callus	2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) 1 mg/l 6(γ,γ-dimethylallylamino)-purine (2ip)
2Z[b]	Shoot regeneration	2 mg/l zeatin, trans isomer
MSS[c,d]	Rooting	none

All media contained salts, sucrose, inositol, pyridoxine HCl, nicotinic acid and agar as in Murashige and Skoog (1962) and 1 mg/l thiamine, except as noted. A solution containing the salts, vitamins, inositol and hormones was adjusted to pH 6.0 before autoclaving. Solutions of agar, sucrose and coconut milk (where used) were autoclaved separately and mixed thoroughly with the balance of the medium before pouring into petri plates.

[a] Also contains 100 ml/l processed coconut milk from mature fruits (Henshaw et al. 1966)

[b] Sucrose concentration reduced to 20 g/l

[c] Agar concentration reduced to 6 g/l; inositol and thiamine deleted;

[d] Pyridoxine HCl and nicotinic acid deleted

Ploidy Determination

The ploidy of the germ cells of the regenerated plants was determined either by measuring the diameter of the mature pollen, the length of the stomata guard cells (Ramulu et al. 1976) or by chromosome counts made on Feulgen-stained pollen mother cells. For breeding purposes it is sufficient that most of the germ cells of the regenerated plants derive from diploid hybrid cells; therefore no special effort was made to detect the chimaeras that are known to sometimes be produced when plants are regenerated from callus (Ramulu et al. 1976).

Electrophoresis

Starch gel electrophoresis and peroxidase staining of leaf tissue extracts were carried out using the recipes of Medina-Filho (1980) and Rick et al. (1977).

Results

Embryo Callus Production

We have never detected fully developed seeds in ripe fruits of the cross (VFNT × LA 1283-4), having checked 30 fruits to date. Further, we have dissected 401 undeveloped seeds from this cross and found no culturable embryos. As an alternative to embryo culture, we decided to try to induce callus from undeveloped seeds, hoping to then regenerate hybrid plants from this callus using standard cell culture techniques.

In the initial embryo callus experiment, approximately 200 pale, white, undeveloped seeds from ripe fruits of the cross (VFNT × LA 1283-4) were plated on 2D/1P CCM medium. These ranged from 1 to 3 mm in their longest dimension. Within the first three weeks, these seeds turned brown and produced a small amount of callus-like growth at the end where the seed had been attached to the fruit. This growth did not continue. Callus that could proliferate indefinitely was produced within two months from the opposite end of six of the seeds. Each callus clone was transferred to a fresh plate of 2D/1P CCM medium as soon as growth was observed. Within 2-3 weeks this callus had grown up and been divided into four pieces 1-2 cm in diameter. Samples from this callus were transferred to 2Z medium. When subcultured at three week intervals, shoots 1-2 cm in length were produced – usually within three months. These shoots were excised from the callus mass and transferred to MSS medium where roots were formed after about two weeks. Clones A, B, D and F produced normal shoots which formed roots and were eventually established in soil. Clones C and E produced very abnormal shoots which never formed roots and were eventually discarded without further study.

The cross (VFNT × LA 1283-4) was repeated in an attempt to determine the optimum time to harvest the

Table 2. Embryo callus production from fruits of various ages

Time after pollination when fruits were harvested[a]	Number of undeveloped seeds plated	Number of seeds that formed callus within 2 months after plating	Per cent of seeds that formed callus
4 weeks, mature green	232	7	3.0%
5-6 weeks, breaker	225	28	12.4%
7 weeks, ripe red	171	1	0.6%

[a] Four fruits were harvested at each time period

Table 3. Ploidy of regenerated hybrid plants

Time after pollination when fruits were harvested	callus clone	Number of plants regenerated	
		diploid	tetraploid
4 weeks (mature green)	1	0	7
	2	0	4
	3	0	9
	4	0	9
	8	–	–
	9	2	0
5-6 weeks (breaker)	5	7	0
	6	–	–
	7	7	0
	10	0	6
	11	0	5
	12	5	7
	31	0	1
	32	0	8
	36	0	6
7 weeks (ripe red)	34	0	5
	A	0	4
	B	3	1
	C	–	–
	D	0	3
	E	–	–
	F	0	3

Numbered clones derive from the experiment shown in Table 2. Clones designated by letters are from the earliest preliminary experiment. Dashes indicate that no normal shoots were recovered from the clone. The ploidy of clone 36 plants was determined by measuring guard cell length, as these plants produced no fully-developed flower buds. The ploidy of all other plants was determined either by measuring pollen diameter or by counting chromosomes

fruits. Table 2 shows that 5-6 week old fruits had the highest percentage of seeds, 12%, forming callus. Table 3 shows that 90% of callus clones from 5-6 week old fruits formed normal-looking shoots that rooted and survived transfer to soil. 40% of these clones had at least one member plant that was diploid. Thus, 4% of the original undeveloped seeds plated eventually yielded diploid plants.

Evidence for Hybridity

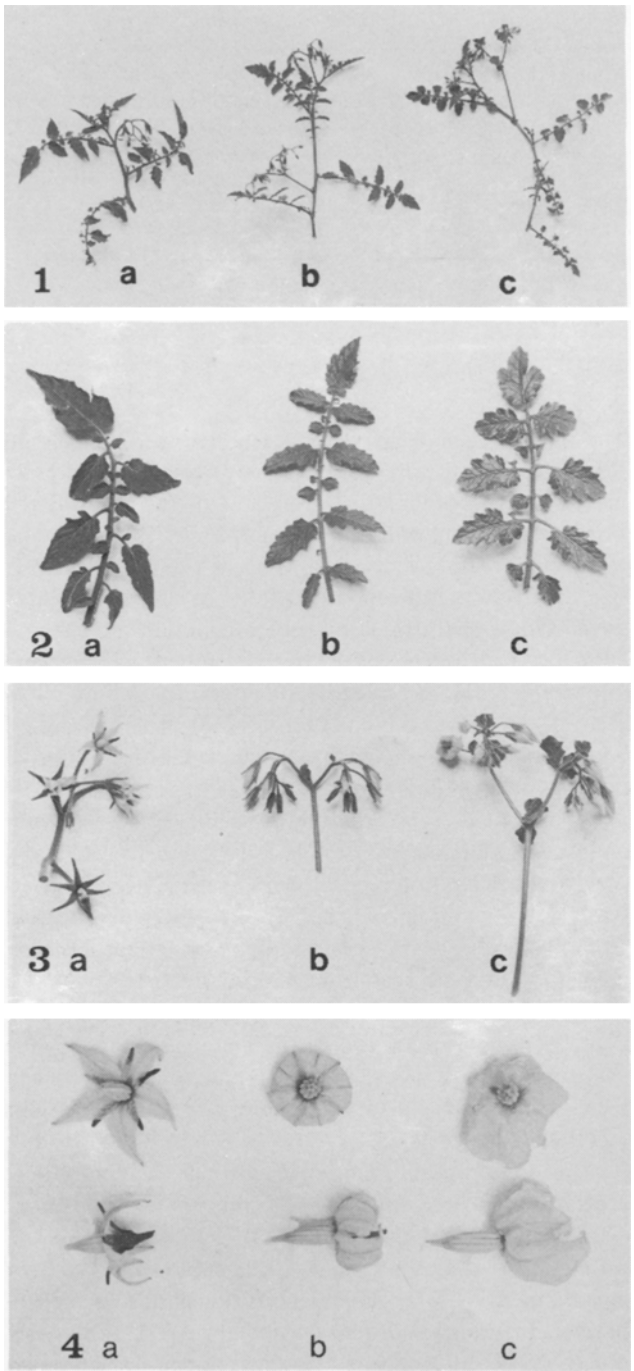
The undeveloped seeds were clearly derived from the intended cross and were not caused by accidental self-pollination or cross-pollination. Over 50% of the emasculated flowers pollinated went on to set fruit. These fruits contained up to 100 seeds each, which is typical for VFNT. Because fruits never contained fully developed seeds, *L. esculentum* pollen from any source could not have stimulated the fruit set observed. No wild tomato stocks were growing in sufficient quantity or proximity to have set so many fruits by wind pollination. The plants were treated with a systemic insecticide to reduce the possibility of insects serving as pollen vectors.

Hybrid seeds are a mixture of hybrid embryo, hybrid endosperm and maternal tissue, but the calli that developed from the hybrid seeds in our experiments were clearly of hybrid embryo origin. As the plants regenerated from the callus were either diploid or tetraploid, they could not have derived from endosperm tissue, which is triploid. Maternal tissue and embryos produced by accidental self-fertilization can be eliminated as sources of the callus by a variety of evidence including callus growth and regeneration rates, plant morphology, fertility relations and isoenzyme patterns.

The embryo callus inherited, virtually undiminished, the rapid callus growth rate and plant regeneration ability of the LA 1283-4 male parent. VFNT callus grows approximately half as fast as does the callus of LA 1283-4 and the hybrids. The plant regeneration ability of the embryo callus must also be inherited from LA 1283-4 because VFNT callus regenerates slowly or not at all under the conditions used.

Morphological traits which show that the plants regenerated from callus were the desired hybrids are pictured in Figures 1-4 and summarized in Table 4.

The fertility relations of the embryo callus-derived plants were exactly those expected of *L. esculentum* x *L. peruvianum* hybrids (McGuire and Rick 1954; Alexander 1963). The regenerated plants were selfsterile, but were able to set fruit having fully developed seeds on other F_1 species-hybrid plants. Fully developed seeds were not formed when the regenerated plants were backcrossed to



Figs. 1a-c-4a-c. Morphology of a VFNT, b embryo callus-derived hybrid and c LA 1283-4

VFNT. (The undeveloped seeds obtained in this backcross were recently plated for embryo callus induction. Seven undeveloped seeds have already produced callus.) If the regenerated plants had derived from maternal tissue in the hybrid seed or from embryos produced by accidental self-fertilization, then the fertility relations expected would be self-fertility, and unilateral incompatibility with F_1 species hybrids (McGuire and Rick 1954).

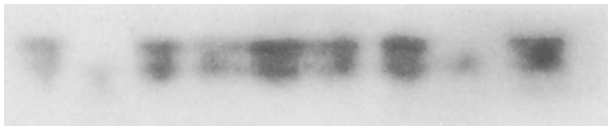


Fig. 5. Peroxidase isoenzyme pattern. Samples from left to right: LA 1283-4, VFNT, hybrid B, hybrid 5, hybrid 7, hybrid 9, hybrid 12, VFNT, 1283-4. Direction of migration from bottom to top

Table 4. Morphological traits of embryo callus hybrids and the parent strains

VFNT	LA 1283-4 and (VFNT X LA 1283-4) hybrids
Stems up to 2 cm in diameter	Stems not exceeding 1 cm in diameter
Leaflets having smooth edges	Leaflets having ruffled edges
Linear inflorescence without bracts	Branched inflorescence having bracts
Fully opened flower	Fully opened flower
stigma inserted in anther cone	stigma exserted from anther cone
sepals prominent	sepals reduced
corolla reduced	corolla prominent
petals fused only at their bases	petals fused over 1/3 of their length
Fruits	Fruits
up to 4 cm in diameter	not exceeding 1.5 cm in diameter
glabrous and red when ripe	hairy and pale yellow when ripe
Dry seeds 3-4 mm long	Dry seeds 1-2 mm long

Starch gel electrophoresis was done to provide further evidence that the regenerated plants are hybrid and that LA 1283-4 actually was the male parent. Figure 5 shows part of a gel stained for peroxidase. LA 1283-4 and the hybrid plants have two strong bands in the Prx-2 region, while VFNT has a single band that comigrates with the slower band of the other plants.

Discussion

Embryo callus-derived plants were demonstrated to be hybrids by a variety of methods. The hybrid seeds showed the characteristic abnormal development and produced callus having a growth rate and plant regeneration ability that was more similar to the male parent than to the female parent. The regenerated plants produced had the morphological traits, ploidy, isozyme banding pattern and fertility relations expected of embryo-derived hybrids.

Although the zymograms provided evidence of hybridity, the genetic basis of the banding pattern can be deduced only from further work. If LA 1283-4 is heterozygous for Prx-2 it is highly unlikely that five independently isolated hybrids would each have inherited the variant allele. A potential explanation of this observation is that LA 1283-4 is homozygous for an allele that covalently

modifies some of the Prx-2 enzyme to produce the fast-migrating enzyme enzyme band observed. Considerable evidence for such a mechanism acting in *L. pimpinellifolium* has been provided by Rick et al. (1979).

Embryo callus is more efficient than embryo culture as a way of producing the *L. esculentum-L. peruvianum* hybrids studied in this paper. Callus was produced in up to 12% of the undeveloped seeds that were plated on callus-inducing medium. Embryos were produced in less than 0.25% of the undeveloped seeds tested. Clearly, few, if any, of the seeds that produced embryo callus could also have yielded culturable embryos.

Although the embryo callus method is useful as is, there remains much potential for improving its efficiency. The timing of media transfers, the composition of the media and the genotype of the *L. esculentum* parent should all be optimized. We plan to make future crosses with *L. esculentum* strains that are homozygous for a recessive seedling marker. Regenerated plants that derive from hybrid cells will not express the recessive marker allele, allowing hybrid plants to be readily distinguished from plants regenerated from maternal tissue or from embryos produced by accidental self-pollination.

We are testing the embryo callus method with other *L. peruvianum* strains as male parents to further test the general usefulness of the method and the media used.

K.E. Scott and M.A. Stevens (personal communication) have applied our embryo callus technique to crosses of *L. esculentum* with *Solanum lycopersicoides* and with *L. chilense*. These crosses produced no fully-developed seeds. Embryos were produced only rarely; development was abnormal and no plants were recovered when these embryos were isolated and submitted to embryo culture. Production of callus from these isolated embryos followed by plant regeneration allowed some hybrids to be salvaged.

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B.R. Thomas
Department of Bacteriology
University of California
Davis, Calif. 95616 (USA)

Note Added in Proof

The first backcross of the original embryo callus hybrids to VFNT has now produced regenerated plants via embryo callus. Embryo callus has also been produced from F_1 hybrids of VFNT and two *L. peruvianum* strains unrelated to LA 1283-4; regenerated plants have already been produced from one of these.