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Development and Differentiation of Haploid *Lycopersicon esculentum* (Tomato)

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Summary. Haploid callus cultures of selected races of *Lycopersicon* (tomato) species can be obtained from anther culture. This is a further demonstration of a proposed general method of haploid culture developed with *Arabidopsis thaliana*. Differentiation of haploid callus of *Lycopersicon esculentum* can be controlled both in the dark and the light by hormones added to defined minimal media. Development to plantlets is achieved only in the light. Callus cells can be induced to develop into seedless pseudo-fruits. Chromosome counts on callus cells or root-tip cells establishes haploidy ($n=12$).

Haploidy can be maintained in culture on defined minimal media for at least one year.

Introduction

What may be a general method for the induction and differentiation of haploid callus and plants from anther culture of diploid angiosperms has been described previously (Gresshoff and Doy, 1972). This method considered (i) the stage of development of the anther at time of removal, (ii) genotypic variation in the potential of an anther to interact with a defined medium to produce haploid plant material, and (iii) that an anther-*via*-callus-to-plant developmental sequence might be more successful than a direct anther-to-plant sequence.

We now present the detailed method of inducing haploid callus from anthers of three races of *Lycopersicon esculentum* (tomato) and the differentiation of haploid callus into various plant structures.

Materials and Methods

Stock Solutions. Mineral salts solution 1 (MS1) (Gamborg and Eveleigh, 1968; Gresshoff and Doy, 1972) contains: $NaH_2PO_4 \cdot H_2O$, 450 mg; Na_2HPO_4, 150 mg; KCl, 1500 mg; $(NH_4)_2SO_4$, 1000 mg; $MgSO_4 \cdot 7H_2O$, 1250 mg; KNO_3, 5000 mg; $CaCl_2 \cdot 2H_2O$, 750 mg; KI, 3.75 mg and distilled water to 1000 ml.

Mineral salts solution 2 (MS2) (Blaydes, 1966; Gresshoff and Doy, 1972) contains: KH_2PO_4, 1500 mg; KNO_3, 5000 mg; NH_4NO_3, 5000 mg; $Ca(NO_3)_2$, 1735 mg $MgSO_4 \cdot 7H_2O$, 175 mg; KCl, 325 mg; KI, 4.0 mg and distilled water to 1000 ml;

Table 1. Composition of growth media

Growth media	Defined basal medium	Hormones	
		NAA (mg/l)	kinetin (mg/l)
1	DBMI	2.0	5.0
2	DBMII	2.0	1.0
3	DBMIII	5.0	0.01
4	DBMII	0.1	—
5	DBMII	0.1	2.0
6	DBMII	5.0	2.0
7	DBMII	8.0	0.1

Growth media 1 to 3 were used for experiments in the dark, media 4 to 7 for experiments with an alternating light-dark cycles as specified in Results, at 27° C ± 1° C and 70% relative humidity.

Trace elements are as Gamborg and Eveleigh (1968).

Iron chelate contains: $FeSO_4 \cdot 7H_2O$, 5.57 g; disodium-ethylene-diamine-tetra-acetate, 7.45 g and distilled water to 1000 ml.

Vitamin-glycine stock solution contains: nicotinic acid, 10 mg; thiamine, 100 mg; pyridoxine, 10 mg; myoinositol, 1000 mg; glycine, 40 mg and distilled water to 1000 ml.

Defined Basal Media. Defined basal medium 1 (DBM1) (Gresshoff and Doy, 1972) was prepared by mixing the following stock solutions: MS1 (200 ml), vitamin-glycine (10 ml), iron chelate (5 ml), trace elements (1 ml), Difco-agar (8 g), sucrose (20 g) and distilled water to 1000 ml. The pH was adjusted to 5.8 to 6.4 with IN NaOH.

Defined basal medium 2 (DBM2) (Gresshoff and Doy, 1972) differs from DBM1 by substituting MS2 for MS1.

Defined basal medium 3 (DBM3) differs from DBM1 in the following: 2% glucose instead of 2% sucrose, the addition of 2 μg/ml biotin and the omission of glycine from the vitamins-glycine solution.

Hormones were added to defined basal media before autoclaving at the concentrations shown in Table 1.

Lycopersicon esculentum Races. All races originated from the N. S. W. Department of Agriculture and were obtained by us from Dr. R. D. Brock of the C. S. I. R. O. Division of Plant Industry, Canberra.

Haploid callus was obtained from anthers of the following races; (Number in brackets indicates C.S.I.R.O. Division of Plant Industry catalogue No.) MHVF145-21-4P (25); VF145-22-8/YTMF8 $BS_2S_1S_6S_1BB$ (27) and No. 65-B-2 (38).

Attempts to obtain haploid callus from anthers of the following forty races were unsuccessful: Rocket (1); Anahu (2); Oahu (3); Hess 6586 (4); Nematex (5); 62B-87 (6); Tropi Gro (7); 7879/M10-10-14 × Atom/7879 (8); No. 3 (9); VFM 10-10-16 (10); Cold Set (11); ES58 (12); Campbell 17 (13); VF206 (14); Yanco Premium (15); YTMF8 (16); Yanco Juice-master (17); Chico Grande (18); Chico (19); K.Y.I. (20); 2003 (21); Long John No. 1 SH (22); VFN 26 (23); VFLOD-12 (24); YRF No. 1 (26); Chinese Red (28); VR Valiant (29); V546 (30); VF Spring Giant (31); Rouge de Marmade (32); Step 286 (33); College Challenger (34); Floralon (35); Indian River (36); Grosse Lisse (37); S. 25 (39); Cl327 × Hardin Miniature (40); Prosperity (41); Manapal (42); VR Rouge de Marmade (43).

Nomenclature. Our nomenclature for tomato callus cell lines was derived as follows: ANU-H25-1 indicates *A*ustralian *N*ational *U*niversity *H*aploid cell line from diploid tomato race *25* (CSIRO Division of Plant Industry catalogue number) cell line number *1*. Thus the other haploid cell lines are ANU-H27-1 and ANU-H38-1. For diploid lines, H would be replaced by D.

Excision of Anthers. The flowers were removed from vigorously growing diploid plants at a bud length of 2–3 mm which usually coincided with a stage of development where the pollen mother cells were in early meiosis, and surface sterilised by immersion in absolute ethanol for 10 seconds. The anthers were removed aseptically (one per flower checked by aceto-orcein squash to determine the stage of meiotic development) placed onto agar slants of sterile growth medium 1 or 2 (10 ml, see Table 1) contained in cottonwool plugged scintillation vials, then incubated at 27° C, 70% relative humidity and 4000 lux m^{-2} for 24 h (to ensure continuation of meiosis as determined by spot tests of a few anthers) after which the light was turned off.

Transfer to growth media 4, 5, 6 and 7 for various types of differentiation was accompanied by incubation in an alternating light-dark cycle.

Chromosome Counts. Actively growing root-tips (or callus) of haploid plantlets were examined by the standard Feulgen method (Darlington and LaCour, 1947).

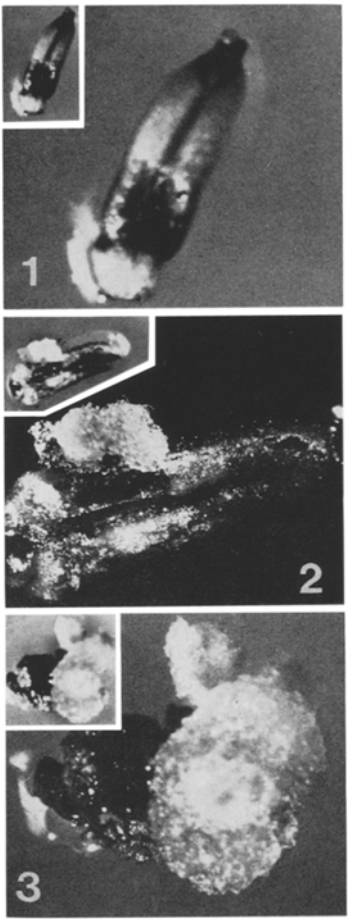
Results

Preliminary studies showed that Gamborg's (Gamborg and Eveleigh, 1968) and Blaydes' (Blaydes, 1966) mineral salts solution best supported diploid callus growth from tomato pith. Therefore these mineral salts solutions were used for DBM1 and DBM2 with the addition of vitamins, trace elements, carbon source and plant growth hormones (see Materials and Methods).

Anther Culture

Forty-three races of *Lycopersicon esculentum* were used to provide anthers. On the basal media and hormonal combinations tested (Table 1) anthers of only three races (25, 27 and 38) produced haploid calluses. Growth media 1 and 2 were optimal for haploid callus induction and growth. The hormonal requirements for callus induction were not specific, as minor variations still produced haploid callus, but at a lower frequency and efficiency. The induction frequency of callus from anthers on growth media 1 and 2 was between 50 and 70%. Callus induction was most successful when the pollen mother cells were still in early meiosis (metaphase I). Anthers which had proceeded beyond the uninucleated pollen grain stage did not produce haploid callus or plantlets under our experimental conditions. Some other races produced swelling and limited growth within the pollen sac, but did not develop sufficiently to be termed callus.

Callus development proceeded as follows: Within a few days after excision the diploid material of the anther browned and then died



Figs. 1—3. Emergence and early growth of haploid callus from *Lycopersicon esculentum* anthers

Fig. 1. An anther before the emergence of haploid callus. It is swollen where callus will emerge and the diploid tissue is brown and dying. Light areas are high-lights not callus

Figs. 2 and 3. Early stages following the emergence of haploid callus. Later the callus is detached from the remains of the anther and sub-cultured

Figs. 1—3 were produced by the rephotography of Kodachrome II transparencies onto Panatomic X. The inserts are to provide a comparison of size under identical conditions of reproduction

(Fig. 1). The pollen sac produced a swelling which, after 4 weeks, burst through the remains of the anther wall exposing clear yellow callus which grew slowly (Figs. 2 and 3). Because of the high kinetin

level (1.5 mg/l) a compact callus was produced. By 8 weeks the callus had reached a fresh weight of 50 to 100 mg and could be subcultured to growth medium 3 (low kinetin, Table 1). The initial doubling time of callus mass on this medium was approximately 14 days. On frequent subculture on growth medium 3 there was a gradual reduction of callus doubling time and at present this is about four days.

Differentiation of Haploid Plantlets

Haploid callus ANU-H27-1 (for nomenclature see Materials and Methods) maintained on growth medium 3 was inoculated onto growth media 4 and 5 (low auxin) and transferred from continuous dark incubation to a 16 h light–8 h dark cycle (Table 1). Within the first week there was a cessation of active growth and slight browning of the callus surface. After 8 weeks greening of the entire callus was complete. Greening did not occur when DBM1 was substituted for DBM2 in growth media 4 or 5. The auxin concentration required for differentiation was specific. A decrease to 0.01 mg/l naphthylacetic acid (NAA) or an increase to 0.5 mg/l NAA prevented greening and subsequent differentiation.

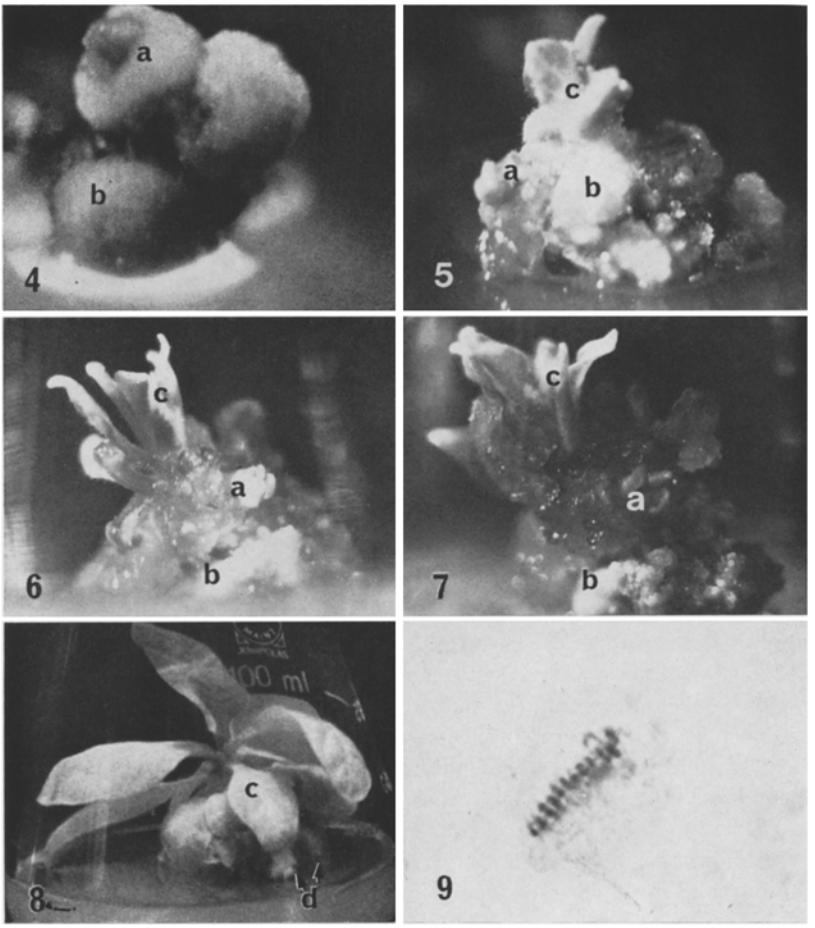
Greening continued until dark green shoot primordia (Fig. 4) arose in depressions on the callus surface. These shoot primordia gave rise to leaf-like structures (Figs. 5—7). In contrast to the differentiation of haploid *Arabidopsis thaliana* plantlets (Gresshoff and Doy, 1972) there was no root development prior to shoot initiation. After 12 weeks, rootless plantlets (about 2 cm height) had developed (Fig. 7). Roots were formed within 2 weeks after transfer to growth medium 7 (high auxin-moderate kinetin, Table 1) and longer periods of darkness (12 hr light-12 hr dark) (Fig. 8). The haploid plantlets have abnormal tomato leaf morphology and have not developed flowers.

Differentiation in the Dark

Haploid callus left in complete darkness (except for observation) on growth medium 5 differentiated into colourless leaf-like structures, which were later overgrown by continued callus growth. This prevented any possibility of plantlet production in the dark.

Development of Callus into „Pseudo-fruits"

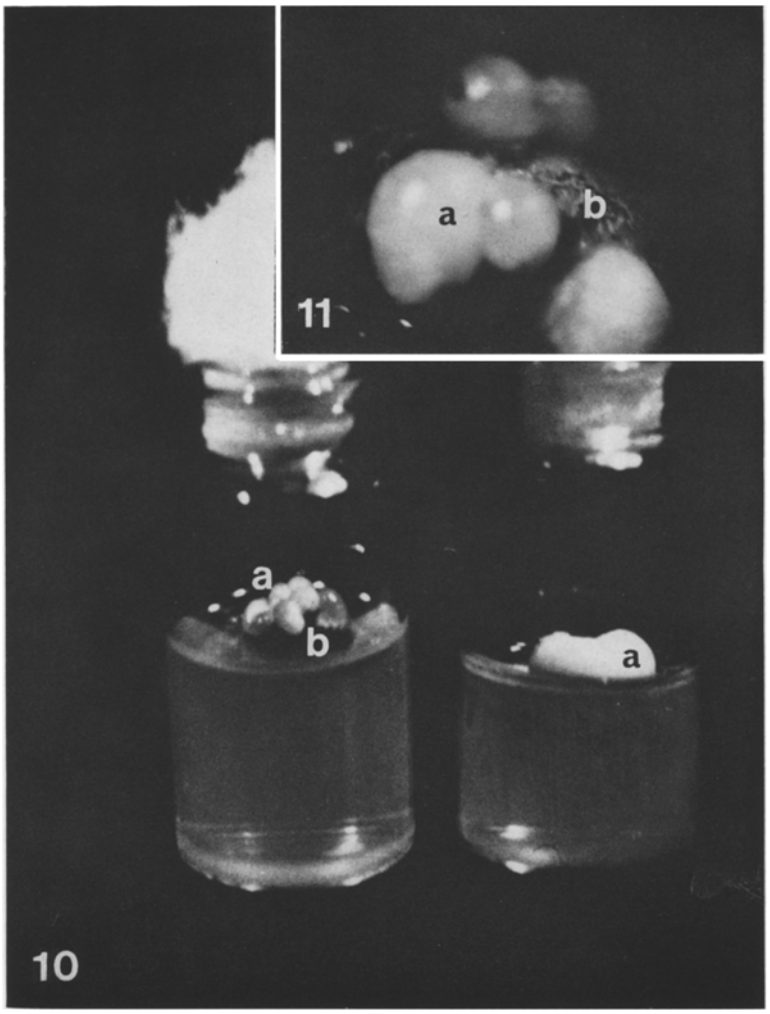
In an attempt to induce rooting at an earlier stage of plantlet development, differentiating calluses were transferred to growth medium 6 (high auxin, moderate kinetin) after 10 weeks incubation and greening on growth media 4 and 5. Instead of roots, green, waxy-skinned pro-



Figs. 4—8a—d. Developmental sequence of *L. esculentum* haploid callus ANU-H27-1 to haploid plantlet (see Results for time sequence). Symbols: shoot primordium (a); callus (b); same plantlet (c) at different stages of development from (a) of Fig. 4; roots (d). If required many plantlets may be developed from the same callus

Fig. 9. Haploid karyotype, early anaphase ($n=12$) from callus squash (see Materials and Methods)

trusions grew from the callus. These stopped growing at a diameter of approximately 1 cm and then gradually "ripened" to dark red (see Figs. 10 and 11). Appearance and taste of these "pseudo-fruits" were similar to more conventionally grown tomato fruits but no seed compartments or seeds have developed.



Figs. 10 and 11. Differentiation of pseudo-fruits from haploid callus. Individual, or groups of developing fruits (a), those shown vary in colour from orange-red to dark red; growth inhibited callus (b). Figs. 10 and 11 were produced by the rephotography of Kodachrome II transparencies onto Panatomic X

Chromosome Counts

Chromosome counts by Feulgen staining demonstrated the haploid state ($n=12$) of both the calluses and the plantlets. After one year of culture on growth medium 3 the calluses remain haploid (see Fig. 9).

Discussion

The selection of a favourable genotype and the correct time of excision of anthers were of crucial importance in the culture of haploid callus from anthers of *Arabidopsis thaliana* (Gresshoff and Doy, 1972) and were of similar importance in the present investigation. The basal salts and other additions were the most effective of a number of fully defined "minimal" media tested (Gresshoff and Doy, 1972). Chemical composition of the media selected was not investigated in detail and may be less than ideal. We have, for example, found that the addition of biotin (2 mg/l, e. g. DBM3, Table 1) stimulates callus growth, particularly if Ion-agar is used. Further stimulation has been obtained by the addition of calcium pantothenate (1 mg/l).

Other workers (Nataka and Tanaka, 1968; Sunderland and Wicks, 1969) have suggested that the uninucleated pollen grain stage is optimal for the successful production of haploid plant cultures. Later developmental stages require higher auxin levels for the induction of mitosis and this induces the division of diploid anther cells (Sunderland and Wicks, 1971). We have found for both *A. thaliana* and *L. esculentum* that it is more convenient to remove anthers in early meiosis and allow them to complete meiosis on the appropriate growth medium (1 or 2, Table 1) for haploid callus induction and growth. On these media there is no induction of growth from diploid anther cells.

In our studies embryoids were not obtained directly from anthers, even on the media successfully used by others for haploid plantlet induction from anthers of *Nicotiana tobacum* and *Datura innoxia* (Guha and Maheshwari, 1966; Sunderland and Wicks, 1969). We conclude that the interaction between different media and plant species is highly specific in determining development. To produce a callus from a uninucleated pollen grain is a less complex developmental sequence than embryoid formation. It is therefore hardly surprising that the anther-callus route has proven successful, while direct embryoid formation has not.

The hormonal requirements in media for differentiation of haploid plantlets from calluses of different plant species varies widely. For example, *A. thaliana* requires relatively high kinetin levels (10 mg/l) but the differentiation of *L. esculentum* does not have an obligate requirement for kinetin, except in the dark (growth media 4 and 5, Table 1). Moreover, 10 mg/l kinetin resulted in death of *L. esculentum* calluses. We have observed that the optimal conditions for differentiation of these two plant species are related to the regulation of callus growth rate by kinetin and auxins (Gresshoff and Doy, in preparation).

As far as we are aware, the induction of pseudo-fruits from undifferentiated callus (whether diploid or haploid) is unique. Clearly it is

not essential to follow a sequence from seed through shoot and flower formation. We postulate that some cells became committed on growth medium 5 (low auxin—moderate kinetin) to a developmental pathway required to form fruits but that this commitment could only be expressed following transfer to a high auxin medium (growth medium 6, Table 1). A moderate kinetin level was retained in order to restrict growth of callus. A similar result has been obtained for a diploid callus culture of *Zea mays*, suggesting that this may be a general phenomenon (Gresshoff and Doy, in preparation).

The present results are an application of methods developed for *Arabidopsis thaliana* anther culture (Gresshoff and Doy, 1972). Success with a second genera encourages the proposal (Gresshoff and Doy, 1972) that the method may have general applicability. Within the genera *Lycopersicon* anther culture is not confined to the species *esculentum*. Haploid calluses were obtained from one of three *Lycopersicum pimpinellifolium* races and one of two *Lycopersicon peruvianum* races.

A progress report on anther culture (Sunderland, 1971) read while this manuscript was in preparation, suggests that *L. esculentum* has been cultured by Sharp. No details were provided and it is not stated if plants were developed directly from pollen or from callus. Other results summarised in this same review support our opinion that it may be possible to widen the success of anther culture by first generating haploid callus.

Our major purpose in developing methods for the production of diploid and haploid callus cultures of higher plants (angiosperms) and for the control of callus differentiation, is to facilitate the study and directed modification of plant genotypes and phenotypes. Haploid cultures represent an extended haploid phase whereby the plant has become a micro-organism which may be amenable to analysis and modification by biochemical and genetical methods. At present we are using *L. esculentum* ANU-H27-1 callus as the plant recipient for phage-mediated transfer and subsequent expression of bacterial genes. Three *Escherichia coli* gene-haploid tomato plant cell systems are under investigation: (i) survival and slow growth of callus on galactose and on lactose as bulk source of carbon as the result of transfer and expression of the galactose and lactose operons respectively; (ii) severe inhibition of callus growth by an amber suppressor gene, *supF*$^+$ (Doy, Gresshoff and Rolfe, 1972a, b).

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