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Genotypic Differences in Shoot-forming Capacity of Cultured Leaf Explants of *Lycopersicon hirsutum*

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Abstract. **Cultured leaf explants obtained from 36 accessions of the wild tomato species, *Lycopersicon hirsutum* Humb. and Bonpl., were evaluated for morphogenic capacity in response to three cytokinins (zeatin, BA, and kinetin) in combination with IAA. Media containing 0.1 μM IAA plus 4.6 or 9.2 μM zeatin were optimal for shoot induction. Cotyledon explants were superior to true leaf explants for obtaining shoot formation. Morphogenic responses of *L. hirsutum* f. *typicum* and *L. hirsutum* f. *glabratum* were clearly accession-dependent and ranged from exceptional with numerous shoots produced to recalcitrant with no shoots produced. The high morphogenetic capacity of leaf explants from *L. hirsutum* f. *typicum* accession 128644 was also evident in protoplast-derived calli that readily regenerated shoots. Chemical names used *(E)*-2-methyl-4-(1H-purin-6-ylamino)-2-buten-1-ol (zeatin), *N*- (phenylmethyl) -1H-purin-6-amine (BA), *N*- (2-furanylmethyl) -*1H*- purin-6-amine (kinetin), *1H*- indole-3-acetic acid (IAA).**

Wild tomato species are a valuable source of economically important traits for introgression into the cultivated tomato *(Lycopersicon esculentum* Mill.). Easily regenerated tomato genotypes facilitate efficient application of cell biological techniques for the transfer of genes controlling these traits. Many reports have described differences in callus and shoot formation from primary explants of cultivars and mutants of

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Table 1. Shoot regeneration of *L. hirsutum* accessions after 8 weeks on culture media containing five combinations of growth regulators.

	Shoots[z]									
	Cotyledon					True leaf				
	Medium[y]									
Accession	A	B	C	D	E	A	B	C	D	E
					L. hirsutum *f.* typicum					
128644	+++(100)	+++(95)	++(60)	++(30)	++(50)	+++(95)	+++(75)	+++(75)	++(25)	+++(75)
127826	+++(85)	+++(75)	++(70)	−(0)	−(0)	++(25)	++(30)	++(40)	−(0)	−(0)
390663	+++(75)	+++(75)	++(30)	−(0)	+(5)	+++(75)	++(25)	+(10)	−(0)	−(0)
126445	+++(95)	+++(95)	++(50)	−(0)	++(25)	++(50)	++(55)	++(60)	−(0)	−(0)
308182	+++(80)	++(25)	+++(90)	−(0)	+(20)	++(30)	+(10)	−(0)	−(0)	−(0)
390661	+++(75)	++(25)	−(0)	−(0)	−(0)	++(35)	++(50)	−(0)	−(0)	−(0)
379010	++(25)	++(30)	++(25)	−(0)	−(0)	+++(75)	++(40)	+(10)	−(0)	−(0)
365934	++(40)	++(40)	+(15)	−(0)	−(0)	++(30)	−(0)	+(10)	−(0)	−(0)
390662	++(30)	++(30)	++(30)	−(0)	+(20)	+(15)	++(25)	+(15)	−(0)	−(0)
127827	++(25)	++(60)	++(40)	−(0)	−(0)	+(20)	+(10)	++(30)	−(0)	−(0)
126446	+(20)	++(40)	++(60)	−(0)	−(0)	++(40)	++(25)	−(0)	−(0)	−(0)
209978	++(60)	−(0)	++(50)	−(0)	−(0)	+(15)	++(25)	+(5)	−(0)	−(0)
390660	+(15)	++(25)	−(0)	−(0)	−(0)	+(15)	−(0)	−(0)	−(0)	−(0)
379014	−(0)	−(0)	++(30)	−(0)	−(0)	++(25)	++(25)	+(15)	−(0)	−(0)
390513	+(15)	−(0)	++(40)	−(0)	−(0)	+(10)	++(25)	−(0)	−(0)	−(0)
390654	++(35)	++(40)	+(20)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
390659	++(25)	+(5)	−(0)	−(0)	−(0)	++(25)	−(0)	−(0)	−(0)	−(0)
390658	++(30)	ND	−(0)	−(0)	−(0)	+(5)	−(0)	−(0)	−(0)	−(0)
390656	−(0)	+(15)	−(0)	−(0)	−(0)	+(15)	−(0)	−(0)	−(0)	−(0)
379012	−(0)	−(0)	+(20)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
379013	+(10)	−(0)	−(0)	−(0)	−(0)	ND	ND	ND	ND	ND
365936	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
390514	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
					L. hirsutum *f.* glabratum					
365904	+++(75)	+++(80)	−(0)	−(0)	−(0)	+++(75)	+++(75)	+(20)	−(0)	−(0)
365905	++(40)	++(30)	++(40)	−(0)	−(0)	++(35)	++(50)	++(35)	−(0)	−(0)
365906	++(70)	++(70)	+(20)	−(0)	−(0)	+(20)	++(25)	−(0)	+(10)	+(5)
365908	++(25)	++(55)	++(25)	−(0)	−(0)	++(30)	+(15)	+(5)	−(0)	−(0)
365907	++(25)	−(0)	+(20)	−(0)	−(0)	−(0)	+(15)	−(0)	−(0)	−(0)
129157	−(0)	+(5)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
126449	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
134417	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
134418	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
199381	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
251304	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
251305	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
365903	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)	−(0)
					L. esculentum					
L-146	+++(80)	+++(75)	−(0)	−(0)	−(0)	+++(75)	+++(75)	−(0)	−(0)	−(0)
Floradade	++(45)	−(0)	−(0)	−(0)	−(0)	++(30)	+(5)	−(0)	−(0)	−(0)

[z]−, no shoot formation; +, poor shoot formation, fewer than five explants formed one to two shoots per explant; ++, good shoot formation, five to 15 explants formed one to five shoots per explant; +++, excellent shoot formation, 15 to 20 explants formed several or more shoots per explant; ( ), percentage of responsive explants; ND, not determined.
[y]Hormone concentrations: (A) 4.6 μM zeatin, 0.1 μM IAA; (B) 9.2 μM zeatin, 0.1 μM IAA; (C) 13.3 μM BA, 1.7 μM IAA; (D) 4.7 μM kinetin, 5.7 μM IAA; (E) 14.0 μM kinetin, 5.7 μM IAA.

tomato (Kurtz and Lineberger, 1983). Evidence for genetic control of shoot regenerability from cultured leaf and callus tissues of *L. esculentum* and *L. peruvianum* has been presented (Koornneef et al., 1987; Tan et al., 1987b). Regeneration of related wild tomato species has focused primarily on *L. peruvianum* (L.) Mill and L. *pennelli* (Koornneef et al., 1987; Tan et al., 1987a). There has been little related research focused on *L. hirsutum* since it is generally regarded as a recalcitrant species for cell culture.

Lycopersicon hirsutum is a heterogeneous species resistant to a considerable range of insect predators, nematodes, and bacterial, viral, and fungal diseases (Taylor, 1986). *Lycopersicon hirsutum is* also a source of genes for cold tolerance, fruit pigmentation, and self-incompatibility (Rick, 1982). This study examines variation among accessions of the wild tomato species, *L. hirsutum*, for regenerability from cultured leaf explants and identifies highly responsive *L. hirsutum* accessions.

Seed of *L. hirsutum* f. *typicum* and *L. hirsutum* f. *glabratum* accessions were obtained from the U.S. Dept. of Agriculture Northeast Regional Plant Introduction Station, Geneva, N.Y. *Lycopersicon esculentum* line L-146 (Michigan State Univ., East Lansing) and *L. esculentum* cv. Floradade were used as *L. esculentum* controls. Seed were surface-sterilized by soaking for 4 min in 70% ethanol, followed by 30 min in a 50% commercial bleach solution and three sterile distilled water rinses. Seed were plated on a modified Murashige and Skoog (MS) medium containing MS salts (Murashige and Skoog, 1962) with supplemental potassium phosphate monobasic and calcium chloride dihydrate (final concentrations: 2.5 and 5.7 mM, respectively), 3% sucrose, 0.01% casein hydrolysate, 0.01% Shepard's (1980) organics, and 0.8% Difco Bacto-agar. Media were adjusted to pH 6.0 before autoclaving for 15 min at 103 kPa. Seed were germinated in a controlled environment chamber at 25C using a 16-h photoperiod (43 $\mu mol \cdot m^{-2} \cdot s^{-1}$) per 8-h dark period.

Fully expanded cotyledons and first true leaves were aseptically removed and trimmed to ≈1.0 × 0.3-cm strips before culturing on duplicate petri plates (100 × 15 mm, 10 explants/plate, 20 explants total/tissue source) containing 25 ml of shoot regeneration media. Shoot regeneration media contained MS salts, 2% sucrose, 0.56 mM *myo*- inositol, 0.30 mM thiamine-HCl, 0.81 mM nicotinic acid, 0.49 mM pyridoxine, and 0.7% agar. The following hormonal combinations were used: (A) 4.6 μM zeatin, 0.1 μM IAA; (B) 9.2 μM zeatin, 0.1 μM IAA; (C) 13.3 μM BA, 1.7 μM IAA; (D) 4.7 μM kinetin, 5.7 μM IAA;

Table 2. Analysis of variance mean squares for shoots formed in *L. hirsutum* f. *typicum* and *L. hirsutum* f. *glabratum*.

	L. hirsutum f. *typicum*			*L. hirsutum* f. *glabratum*		
Source	df	Mean square	F	df	Mean square	F
Media (M)	4	0.82	20.5**	4	0.14	4.7**
Tissue (T)	1	0.50	12.5**	1	0.02	2.0
Accession (A)	22	0.34	17.0**	12	0.12	12.0**
M × T	4	0.07	3.5*	4	0.01	1.0
M × A	88	0.04	2.0**	48	0.03	3.0**
T × A	22	0.04	2.0*	12	0.01	1.0
Error	88	0.02		48	0.01	

*,**Significant at $P = 0.05$ or 0.01, respectively.

(E) 14.O μM kinetin, 5.7 μM IAA. Media were adjusted to pH 5.8 before autoclaving for 15 min at 103 kPa. The IAA solution was filter-sterilized and added aseptically to the media after autoclaving. Growth regulators and concentrations used were selected for their reported capacity to induce shoot formation in L *esculentum* (Kurtz and Lineberger, 1983; Locy, 1983; Tan et al., 1987b; Uddin et al., 1988) and on the basis of preliminary observations for shoot induction in *L. hirsutum* (data not shown). All plates with explants were distributed in a random fashion and incubated as described for seed germination. Recognizable shoots were scored at 4-week intervals. Only data recorded at 8 weeks are presented. No increases in shoot count were noticed after 8 weeks of culture.

For protoplasm isolation, seedlings of L *hirsutum* f. *glabratum* accession 128644 were grown from seed as described above. At 12 days, seedlings were cut at the hypocotyl and the tips transferred to Plant-Cons (Flow Laboratories, McLean, Vs.) containing fresh seed germination medium. Three-week-old axenic seedlings were preconditioned before protoplasm isolation by placing them in the dark at 10C for 12 h. Protoplasts were isolated using the methods described by Niedz and Sink (1988) with minor modifications. Preconditioned true leaves were excised, sliced transversely into l-mm strips, and digested in enzyme solution [1.0% (w/v) cellulysin, 0.1% macerase, and 0.4 M sorbitol in CPW salts (Frearson et al., 1973) at pH 5.7] for 4 h at 27C. Enzyme digests were filtered through cheesecloth, pelleted by centrifugation (35 g, 10 rein), and floated twice on a 17% sucrose solution (100 g, 10 rein) before plating. Protoplasts were cultured according to the methods described by Shepard (1980). Protoplasts were plated (0.5×10^5 protoplasts/ml) in Shepard's cell layer (CL) medium containing 25% of the usual salts in a CL reservoir plate. About 40 microcalli were transferred to Shepard's C medium after 14 days and then to Shepard's D medium after 30 days for regeneration.

Significant differences in morphogenetic potential of cultured leaf explants were observed among the L *hirsutum* accessions studied (Tables 1 and 2). Analysis of shoot counts suggested ranking the accessions according to the number of shoots produced and the percentage of explants giving rise to shoots. Hormones in the culture medium had a significant effect on shoot formation for both *L. hirsutum* f. *typicum* and *L. hirsutum* f. *glabratum* (Table 2). Among the combinations of hormones tested, 4.6 μM zeatin plus 5.7 μM IAA was optimal for shoot induction in most accessions. Accessions performing well on zeatin-based media produced either a similar number of shoots or fewer on media containing BA as the cytokinin. Media supplemented with kinetin plus IAA induced little or no shoot regeneration from explants of *L. hirsutum*, confirming the results of Locy (1983). In contrast, kinetin plus IAA has been reported to be similar to zeatin in shoot formation from leaf explants of *L. esculentum* cultivars (Uddin et al., 1988). Independent of hormones in the medium, L *hirsutum* f. *typicum* accession 128644 displayed a high frequency of shoot regeneration, particularly from true leaf explants.

In comparison to true leaf explants, cotyledons of *L. hirsutum* f. *typicum* were generally superior explant sources for shoot regeneration with the five hormone combinations examined. No significant tissue effects were evident for *L. hirsutum* f. *glabratum* (Table 2). High shoot-forming capacity of cotyledon tissue has been noted for *L. esculentum* (Fillatti et al., 1987).

Included among *L. hirsutum* f. *typicum* accessions that regenerated the most shoots were 128644, 127826,390663, and 126445. Number of shoots and percentage of responsive explants produced by these accessions on zeatin-containing media were higher than or similar to *L. esculentum* line L-146, a cherry-type tomato generally regarded as superior in shoot regenerability. Relative to L *esculentum* 'Floradade', these *L. hirsutum* accessions exhibited superior regenerability on the media tested. In contrast, accessions such as 390656, 365936, and 390514 displayed little or no morphogenetic capacity under these conditions. Locy (1983) found little or no shoot regenerability among explants of a single *L. hirsutum* accession tested.

Accessions 365904,365905,365906, and 365908 were among the most highly responsive of *L. hirsutum* f. *glabratum* examined for shoot formation. Similar to highly responsive *L. hirsutum* f. *typicum* accessions, 365904 performed similar to line L-146. Overall, *L. hirsutum* f. *glabratum* exhibited a higher percentage of recalcitrant accessions than *L. hirsutum* f. *typicum* and was less responsive for shoot regeneration on the media tested (t test significant at $P = 0.01$). A previous study (Kut and Evans, 1982), limited to examination of two *L. hirsutum* f. *glabratum* accessions, found only a single regenerate after 13 weeks of culture.

Supporting our initial positive observations on shoot regenerability from *L. hirsutum* cotyledon and true leaf explants, cultured leaf mesophyll protoplasts of the highly responsive *L. hirsutum* f. *typicum* accession 128644 readily gave rise to shoots. Although plating efficiency of protoplasm preparations was low (< 1%; $\approx 1.2 \times 10^3$ viable protoplasts/1.25×10^5 plated protoplasts), surviving microcalli exhibited a relatively high frequency of shoot production when placed on regeneration medium (regeneration efficiency = 65%; 26 shoot-forming microcalli/40 cultured microcalli). A positive correlation between shoot regeneration from leaf explants and from mesophyll protoplasts has been demonstrated in *L. esculentum* (Tan et al., 1987). A similar relationship may be suggested by our limited results for cultured *L. hirsutum* protoplasts. Using leaf explant regenerability scores to identify *L. hirsutum* accessions with high regenerative capacity from protoplasts can facilitate genetic manipulations that use protoplasts as intermediary steps.

Although generally regarded as a recalcitrant wild tomato species, *L. hirsutum* accessions were identified that are similar or superior to *L. esculentum* for shoot regenerability from cultured explant tissues. The degree of regenerability in *L. hirsutum* accessions ranged from exceptional with numerous shoots produced to recalcitrant with no shoots produced, suggesting genetic variability for shoot-forming capacity.

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