Detection of Citrus Exocortis Viroid by *In Vitro* Inoculation of Callus Culture

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ABSTRACT. Citrus exocortis viroid is one of the major diseases limiting production of citrus in China. A new exocortis detection system was developed using calli cultured *in vitro* as indicators. The callus of Etrog citron 861-S-1 inoculated with CEV showed a significant growth reduction and turned brown in 10 to 15 days. CEV concentration had no significant effect on symptom expression but symptoms were much better at an alternate temperature regime of 30-35°C than at a constant 26, 30, or 35°C. Calli of several citrus species and hybrids were tested for exocortis sensitivity *in vitro*. Differences between species were similar to those observed in the field. The new inoculation procedure could be used for quick selection of somatic hybrids with resistance in a breeding program to obtain scion and rootstock varieties resistant to exocortis.

Key words. CEV, resistance.

Citrus is one of the most important fruit crops in the world, and China has a significant citrus gene bank with many cultivars and wild citrus types. With worldwide germplasm exchange, virus and virus-like diseases have been introduced to the Chinese citrus industry. Presently, citrus exocortis viroid (CEV) is one of the most important pathogens affecting citrus production in China. Along the reaches of the Yangtse River, there is a major citrus production area where trifoliate orange is the most important rootstock for satsuma and sweet orange trees. However, this rootstock is sensitive to exocortis and, as mentioned above, the disease has caused serious losses. Early identification of infected trees and use of certified pathogen-free budwood and exocortis-resistant rootstocks and scions are important measures to reduce disease damage.

In 1989, we started a project aimed at obtaining CEV-resistant citrus by somatic hybridization. In a first step, we decided to use callus cultures to detect virus diseases *in vitro* and use this procedure for quick selection of CEV-resistant cell lines. The results of this preliminary research are reported here.

Calli were obtained from Etrog citron 861-S-1, Swingle citrumelo, Ichang papeda, Satian pummelo, sour orange, Troyer citrange and trifoliate orange. For primary callus culture, the basal medium of Murasighe and Tucker (1) was supplemented with 3% sucrose, 1.5 mg/liter 2,4-dichloro, phenoxiacetic acid (2,4-D), 1.0 mg/liter naphtalenacetic acid, 0.15 mg/liter kinetin and 0.1 mg/liter zeatin. For subculturing media, 2,4-D was reduced from 1.5 mg/liter to 1.0 mg/liter. The calli were incubated under permanent cool fluorescent light (1,500-2,000 lux) and several temperatures were assayed to optimize symptom expression.

The CEV sources assayed for inoculation included: 1) A mild isolate from Jingchen 73-10 sweet orange; 2) CEV-infected Java gynura calli offered by the Wuhan Virology Research Institute; and 3) CEV-infected Etrog citron 861-S-1 and navel orange trees from Hunan province, identified by the Hunan Horticultural Research Institute by symptom expression of indicator plants. The inoculum consisted of total nucleic acid extracts, or extracts concentrated by 2M LiCl precipitation. All CEV extracts were filter sterilized (0.45 nm) prior to inoculation.

Callus culture of Etrog citron 861-S-1 was a good indicator of CEV *in vitro*. Infected calli showed signifi-

TABLE 1
EFFECT OF INOCULATION OF ETROG CITRON 861-S1 CALLUS CULTURE WITH TWO ISOLATES OF CITRUS EXOCORTIS VIROID

Viroid isolate	Symptom in calli	Appearance of symptoms	Growth of calli (g)		Growth reduction (%)
			Uninoculated control	Viroid inoculated	
Severe CEV	Browning	10 days	3.37 a	1.89 b	43.02
Mild CEV	Browning	15 days	3.23 a	2.03 b	37.15

Means followed by a different letter in a row are significantly different ($P < 0.01$).

cantly reduced growth and browning 10 to 15 days after inoculation (Table 1). Concentration of CEV extracts had no significant effect on symptom expression, but this was affected by the incubation temperature. An alternating temperature regime of 30°C (12 h/day) and 35°C (12 h/day) induced better symptoms than a constant temperature of 26, 30 or 35°C. Three inoculation procedures were assayed for callus inculcation: 1) cutting with a knife dipped in extract;, 2) soaking in extract; and 3) cutting and soaking. Cutting and soaking gave the highest infection rate (85 to 100% of the inoculated calli).

It was observed that several tubes of CEV-infected calli which had turned deep brown yielded calli of normal white color after subculture for 10 days. This phenomenon needs further study.

The new *in vitro* inoculation system was used with calli obtained from several citrus species and hybrids to test their relative resistance to CEV (Table 2). It was observed that Swingle citrumelo, Ichang papeda, Satian pummelo and sour orange were more resistant than Troyer citrange and trifoliate orange. Sensitivity of these varieties to CEV *in vitro* was similar to that observed under field conditions.

Etrog 861-S-1 callus culture was a good indicator for CEV detection *in vitro* due to its quick growth and white color. Browning of infected calli occurred 10 to 15 days after inoculation and allowed easy differentiation of these from uninfected calli which had a white color. This shortened the detection period from several months in biological assays with citron indicators to 15 days and has a great advantage for practice use. The procedure is cheap and easy to perform if a permanent callus culture is maintained in the laboratory. The fact that calli from several species and hybrids were affected by CEV inoculation according to their sensitivity in the field suggests that this inoculation procedure could be

TABLE 2
CHANGES IN GROWTH OF CITRUS CALLI AFTER INOCULATION WITH CEV

	Cultivar of calli[z] (g)					
	Swingle citrumelo	Ichang papeda	Satian pummelo	Sour orange	Troyer citrance	Trifoliate orange
Uninoculated control	1.90	2.30	1.76	2.60	3.09	2.71
Inoculated with CEV	1.77 a	1.96 a	1.37 a	1.90 a	1.75 ab	1.13 b
Reduction (%)	6.84 a	14.78 a	22.16 a	24.20 a	43.48 ab	53.51 b

[z]Mean growth in g obtained from 22 culture tubes per treatment.
Means followed by a different in a column are significantly different ($P < 0.01$).

useful for quick selection *in vitro* of somatic hybrids with resistance to CEV. This can be very helpful to speed up the Chinese breeding program to obtain CEV-resistant scion and rootstock varieties.

LITERATURE CITED

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