RESEARCH NOTE

The Elimination of Fanleaf Virus from Grapevines Using *in vitro* Somatic Embryogenesis Combined with Heat Therapy

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Somatic embryos were successfully regenerated from callus tissue of anthers and ovaries excised from inflorescences of grapevines infected with grapevine fanleaf virus (GFLV). Production of pro-embryogenic masses (PEMS) was controlled by specific growth regulators and culture conditions, including heat incubation at 35°C. Somatic embryos (containing roots and cotyledons) and plantlets were subjected to immunosorbent electron microscopy (ISEM) and serological tests (ELISA). Results show that somatic embryogenesis in combination with heat therapy of the cultures is an effective procedure to eliminate GFLV from anther and ovary source material.

Grapevine fanleaf virus (GFLV) is the most important and most widespread virus of grapevines (Hewitt *et al.*, 1970; Goheen, 1988; Bovey & Martelli, 1992). Since the GFLV is spread mainly through infected propagation material, the establishment of vineyards with vines free of the fanleaf virus is the primary control measure (Goheen, 1988).

Heat therapy of infected vines followed by *in vitro* shoot apex cultures has been used successfully to eliminate fanleaf virus from grapevines (Gifford & Hewitt, 1961; Bass & Vuittenez, 1977; Harris & Stevenson, 1979; Monette, 1986). Regeneration of GFLV-free vines was reported by Barlass *et al.* (1982), utilizing the fragmented apex technique in combination with heat therapy of the cultures. The elimination of leafroll-associated viruses from grapevines using *in vitro* somatic embryogenesis was successfully achieved (Goussard *et al.*, 1991) but the procedure was not effective in eliminating GFLV.

This paper reports on the regeneration of somatic embryos from grapevine material with fanleaf symptoms and demonstrates that GFLV could be eliminated by the use of this technique in combination with heat therapy.

MATERIALS AND METHODS

Plant material: Dormant canes of *Vitis vinifera* L. cv. Gewürztraminer and *Vitis rupestris* cv. Rupestris du Lot with severe symptoms of yellow mosaic and fanleaf, respectively, were collected from field-grown vines. The canes were cut into lengths of *ca* 40 cm, treated with Captan (2%) and stored in sealed plastic bags at 3°C.

Large quantities of elongating shoots were procured using the method of Goussard (1981). Flower development on shoots bearing inflorescences was promoted by the removal of all vegetative tissues (Mullins, 1966). Inflorescences were harvested and chilled (72 h) at 4°C when the anthers of individual flowers were translucent yellow-green in colour.

Regeneration of somatic embryos and heat therapy: Anthers and ovaries used as explants were aseptically excised from flower buds of Rupestis du Lot and Gewürztraminer, respectively. The induction medium consisted of the basal medium (BM) of Nitsch & Nitsch (1969). For culturing ovaries, Nitsches' medium was supplemented with 6-benzyl amino purine (BAP; 5 μM,), 2,4-dichlorophenoxyacetic acid (2,4-D; 2,5 μM) and ß-naphthoxyacetic acid (NOA; 2,5 μM), whereas for anther culture only BAP (1 μM) and 2,4-D (5 μM) were added to the BM. Twenty explants were cultured per 100 ml flatbottomed glass jar with transparent lid. Each jar contained 15 ml of liquid medium.

Immediately after preparation the cultures (two sets of four jars containing anthers and ovaries of Rupestris du Lot and Gewürztraminer, respectively) were incubated in darkness and constantly agitated on an orbital shaker (80 rpm) at 35°C. The same procedure was followed for incubation at 25°C and culturing procedures were as described previously (Goussard *et al.*, 1991). Heat treatment at 35°C was terminated after 60 days and the cultures were transferred to BM (hormone-free) and grown at 25°C. Mature embryos were germinated (Goussard *et al.*, 1991) and somatic plantlets acclimatized and transferred to soil using the method described by Goussard & Wiid (1989).

Virus detection: The virus status of source material and of regenerated somatic embryos and plantlets was established by subjecting samples to both immunosorbent electron microscopy (ISEM) with decoration (Milne *et al.*, 1984) and enzyme-linked immunosorbent assay (ELISA) (Clark & Adams, 1977).

RESULTS

Callus growth and somatic embryogenesis: At both temperature regimes moderately fast-growing callus aggregates were formed. In some cases the constant agitation caused the disintegration of callus aggregates after 15 days of culture.

Following transfer to a solidified growth-regulator-enriched medium, noticeable differences in culture growth were observed between treatments at 25°C and 35°C. At the lower temperature the development of pro-embryogenic masses (PEMS) required transfer of calli to a hormone-free medium, whereas at 35°C nodular callus formed PEMS (densely packed with white embryoids) directly in media amended with growth regulators, without the development of granular watery structures. These embryoids, however, did not elongate beyond the heart stage of embryonic growth. In culturing at 25°C PEMS appeared as isolated spots surrounded by brown/black nodular callus.

After continued subculturing (without hormones and after heat incubation had been terminated) embryoids developed into somatic embryos. Mature embryos containing expanded cotyledons and elongated roots formed at *ca* 70 days after the start of culture from heat-treated explants. Without heat therapy a longer period (*ca* 90 days) was needed.

Embryos derived from anthers and ovaries gave rise to vigorously growing plants displaying the normal characteristics of the grapevine. However, plantlets regenerated from cultures grown at 25°C revealed typical symptoms of the fanleaf disease (Bovey & Martelli, 1992) whereas heat incubation of cultures produced plants free from fanleaf symptoms (Fig. 1).

Virus detection: Results of ELISA tests and ISEM performed on source material as well as somatic embryos and plantlets are presented in Table 1. These tests confirmed the presence of GFLV particles in field-grown Gewürztraminer and Rupestris du Lot. All tested somatic embryos and plantlets derived from cultures that were subjected to heat therapy (35°C for 60 days) were negative for GFLV. Embryos and plantlets produced without heat therapy (25°C) were all still infected with fanleaf virus.

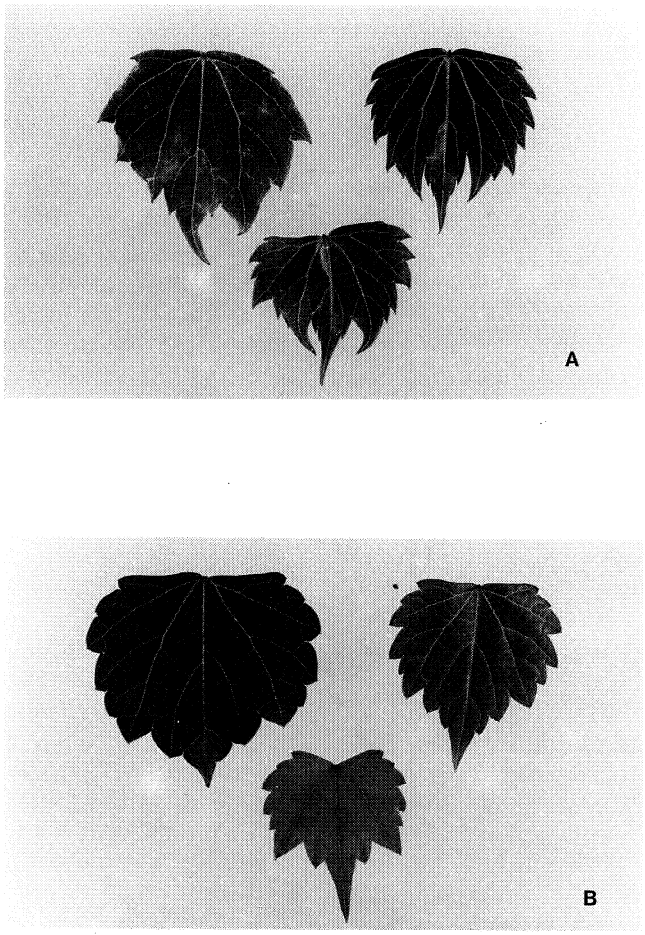

FIGURE 1

Leaf characteristics of somatic plantlets derived from anthers of Rupestris du Lot infected with fanleaf virus. Leaves in A (cultures grown at 25°C) reveal typical symptoms of the fanleaf disease whereas leaves in B (cultures grown at 35°C) are free from fanleaf symptoms.

TABLE 1
Indexing of Gewürztraminer and Rupestris de Lot source material and regenerated somatic embryos and plantlets by ISEM and ELISA.

Virus type	Source material *				Somatic embryos and plantlets **							
	Gewürztraminer		Rup du Lot		Gewürztraminer				Rup du Lot			
	ISEM	ELISA	ISEM	ELISA	ISEM		ELISA		ISEM		ELISA	
					1	2	1	2	1	2	1	2
GFLV	+	+	+	+	+	–	+	–	+	–	+	–
GFLV (Yellow mosaic serotype)	+	+	+	+	+	–	+	–	+	–	+	–

1= Cultured at 25°C.
2= Cultured at 35°C.
* Tests were performed on material sampled from one source vine.
** In each cultivar tests were performed on 10 somatic embryos and 10 plantlets selected at random; + = virus detected; – = virus not detected.

DISCUSSION

The potential of *in vitro* somatic embryogenesis for eliminating GFLV from grapevines has been indicated. Use of this technique, which is based on the production of somatic plantlets via callus tissue from anthers and/or ovaries infected with grapevine leafroll-associated viruses, has itself produced grapevines free from these viruses (Goussard *et al.*, 1991). Results of the present investigation confirm that somatic embryogenesis combined with heat therapy of the cultures is an effective technique to eliminate fanleaf virus from grapevines.

The presence of GFLV particles in plantlets regenerated by somatic embryogenesis without heat incubation indicated that these viruses were translocated from infected tissue via proliferating callus (lacking vascular tissue) to embryoids. This would support the findings of Reynolds & Corbett (1980) and Barlass *et al.* (1982) that fanleaf virus particles are not restricted to vascular tissue and are present in very young meristematic tissues (i.e. meristematic domes of shoot apices), thus allowing translocation to callus and to subsequently regenerated embryoids.

The present report is the first to show effective elimination of fanleaf virus from grapevines using *in vitro* somatic embryogenesis in combination with heat therapy of the cultures. The technique has great potential for eliminating leafroll-associated viruses as well as NEPO viruses from the same source vine. Research on the elimination of grapevine fleck virus [a grapevine phloem limited isometric virus (GFLIV) (Boscia *et al.*, 1991)] using somatic embryogenesis, is in progress.

This technique could be advantageously applied for: (i) the elimination of GFLV as well as leafroll-associated viruses from grapevines utilizing only one procedure, (ii) the production of good quality indicator material and (iii) the establishment of commercial cultivars for reinfection with single or other combinations of virus isolates for research purposes.

It is further postulated that this technique could also be effective in eliminating other NEPO viruses normally eliminated by heat therapy.

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