

The Effectiveness of *in vitro* Somatic Embryogenesis in Eliminating Fanleaf Virus and Leafroll Associated Viruses from Grapevines

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Somatic embryos were successfully regenerated from callus tissue of anthers and ovaries extracted from inflorescences of grapevines infected with grapevine fanleaf virus (GFLV) and grapevine leafroll associated viruses (GLR) respectively. Production of pro-embryogenic masses (PEMS) was controlled by specific growth regulators and culture conditions. Somatic embryos (containing roots and cotyledons) and plantlets were subjected to immunosorbent electron microscopy (ISEM) as well as serological tests (ELISA). Results indicated that somatic embryogenesis derived from ovary tissue of infected grapevines is an effective technique to eliminate grapevine leafroll associated viruses from grapevines but the procedure was not successful in the elimination of GFLV from anther source material.

Grapevine leafroll (GLR) disease and grapevine fanleaf virus (GFLV) are common in South African vineyards and of great importance to growers, nurserymen and winemakers. Closterovirus-like particles have been detected in grapevine material showing leafroll symptoms (Namba *et al.*, 1979; Faoro *et al.*, 1981; Castellano *et al.*, 1983; Zee *et al.*, 1987) while GFLV-particles (Hewitt *et al.*, 1970) are present in grapevines showing fanleaf symptoms.

In vitro shoot apex cultures have been used successfully to eliminate harmful viruses from grapevines (Mur, 1979; Barlass *et al.*, 1982; Jákó, 1988). Somatic embryogenesis in the grapevine (Mullins & Srinivasan, 1976; Krul & Worley, 1977; Rajasekaran & Mullins, 1979, 1983; Srinivasan & Mullins, 1980; Bouquet *et al.*, 1982) has a great potential for plant improvement; however, reports are lacking on the use of this technique for the elimination of viruses from grapevine material.

This paper reports on the generation of somatic embryos from grapevine material showing leafroll and fanleaf symptoms and to determine if these viruses could be eliminated by the use of this technique.

MATERIALS AND METHODS

Plant material: Dormant canes of *Vitis vinifera* L. cv. Roobernet (Cabernet Sauvignon x Pontac) and *Vitis rupestris* cv. Rupestris du Lot showing severe symptoms of leafroll 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 2 - 3°C.

Large quantities of elongating shoots were procured according to the method described by Goussard (1981). Flower development on shoots bearing inflorescences was promoted by removal of all vegetative organs (Mullins,

1966). Inflorescences were harvested when the anthers of individual flowers were translucent yellow-green in colour, and they were then chilled (72h) at 4°C.

Regeneration of somatic embryos: The inflorescences were surface-sterilized with calcium hypochlorite (7% available chlorine) containing Tween 20 (0,1%) as a wetting agent. Anthers and ovaries were excised from flower buds of Rupestris du Lot and Roobernet respectively and used as explants.

Ovaries of Roobernet were cultured in Nitsch & Nitsch (1969) medium supplemented with cytokinin {6-benzyl amino purine (BAP; 5 µM)} in combination with auxins {2,4-dichlorophenoxyacetic acid (2,4-D; 2,5 µM) and β-naphthoxyacetic acid (NOA; 2,5 µM)}. In culturing anthers, Nitsches' medium was enriched with BAP (1 µM) and 2,4-D (5 µM) without the addition of NOA. Twenty explants were cultured per 100 ml flatbottomed glass jars with transparent lids and each jar contained 15 ml of liquid medium. The cultures were constantly agitated on an orbital shaker (80 rpm) at 25°C in darkness. After 1 month cultures were transferred to 100 mm diameter petri dishes containing the same medium solidified with 0,7% Difco-Bacto agar and cultured for 30 days.

To express somatic embryo formation, the callus tissues resulting from the first two months' culture were transferred to agar-solidified basal medium (BM) of the same nutrient composition as the induction medium but without growth regulator supplements. Cultures were incubated on BM, with monthly transfers, until mature embryos containing cotyledons and roots were formed. Germination was achieved by transferring mature embryos to solid BM in glass jars under light incubation. Somatic plantlets were acclimatised and transferred to soil using the method described by Goussard & Wiid (1989).

Virus detection: The virus status of source material and of mature 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) with available kits (Clark & Adams, 1977).

ISEM: Immunosorbent electron microscopy for the detection of grapevine leafroll associated viruses was performed according to the method described previously (Goussard *et al.*, 1990). Samples for the detection of GFLV by this method were prepared by grinding young actively growing shoot tips and leaves as well as mature somatic embryos in 0.1 M phosphate buffer with 2% polyvinylpyrrolidone (PVP), omitting the liquid nitrogen step.

ELISA: The detection of grapevine virus A (GVA) and grapevine leafroll associated viruses I, II and III was done as described by Gugerli (1987) and Zee *et al.* (1987). GFLV was detected according to Clark & Adams (1977), using an ELISA kit prepared locally for the detection of GFLV.

RESULTS

Callus growth and somatic embryogenesis: Explants formed moderately fast-growing callus aggregates. In some cases the constant agitation caused the disintegration of callus aggregates after 15 days of culture. On transfer of the cultures to solid growth regulator enriched medium, the callus turned dark yellow/brown and a specific type of callus tissue developed. It consisted mainly of large rounded non-friable nodules with a brown colour. Maintained on growth regulator enriched media, the nodules continued enlarging. Fresh nodules that formed out of the main nodules displayed a light yellow colour which darkened as the nodules matured. In the presence of growth regulators, nodule enlargement continued but somatic embryos did not develop. Within 14 days of transfer to BM, slightly granular watery callus clusters (pro-embryogenic masses, PEMS) developed out of the nodules (Fig. 1). As maturation advanced, the granular texture became more apparent and individual white nodular structures appeared. These structures developed into somatic embryos with subsequent cotyledon expansion (Fig. 2). Roots covered with fine root hairs formed shortly after cotyledon expansion. Mature somatic embryos containing well developed cotyledons and roots (Fig. 3) developed ca. 90 days after the start of culture.

Germination and plantlet formation: Mature embryos were transferred to glass jars containing BM and placed under subdued light conditions for 7 days, after which the cultures received a photoperiod of 16 h and an irradiance of $53 \mu\text{E m}^{-2} \text{sec}^{-1}$. Germination of somatic embryos (the formation of apical meristems followed by shoot elongation) occurred within 14 days (Fig. 4). The germinated embryos grew rapidly and on reaching the lid of the culture bottles, developing plantlets were removed from sterile culture conditions, acclimatised and transferred to soil. Ovary- and anther-derived embryos gave rise to vigorous growing plants displaying the normal morphological characteristics of the grapevine (Fig. 5).

Virus detection: Electron microscopy (ISEM) of phloem sections from Roobernet cuttings revealed the presence of GVA, grapevine leafroll associated viruses II and III, an unidentified spherical virus-like particle as well as an undecorated closterovirus particle (Fig. 6). GFLV-particles were present in the preparation made from actively growing leaves and shoot tips of Rupestris du Lot (Fig. 7). 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 grapevine leafroll associated viruses and GFLV-particles in field-grown Roobernet and Rupestris du Lot, showing severe leafroll and fanleaf symptoms respectively. The regenerated embryos and plantlets (selected at random) from ovaries of GLR-infected Roobernet tested negative for all grapevine leafroll associated viruses present in the source material, but regenerated embryos and plantlets from anthers of GFLV-infected Rupestris du Lot were still infected with GFLV.

DISCUSSION

The regeneration of somatic embryos from grapevine material infected with GLR and GFLV respectively was accomplished successfully. In most cases (Mullins & Srinivasan, 1976; Krul & Worley, 1977; Rajasekaran & Mullins, 1979; Srinivasan & Mullins, 1980) the induction of somatic embryogenesis in the grapevine appears to be dependent on the presence of BAP and auxin in the culture medium. In the present investigation, embryos were produced from callus formed by cultured ovaries of Roobernet (infected with GLR) and anthers of Rupestris du Lot (infected with GFLV) on induction media supplemented with BAP in combination with 2,4-D and NOA. This is in accordance with the findings of Newton (1990) in culturing ovaries and anthers of *Vitis vinifera* L. cv. Cabernet Sauvignon.

In detailed studies on the ontogeny of grapevine somatic embryos, no vascular connections between any two neighbouring embryoids or between embryoids and the parent tissue could be observed (Newton & Goussard, 1990). In the present investigation, the absence of grapevine leafroll associated viruses in regenerated embryos and plantlets indicates that there was no translocation of these viruses from infected tissue via proliferating callus (without vascular tissue) to embryoids. This would support the findings of Namba *et al.* (1979) that closterovirus particles are restricted to vascular tissue (phloem). It was clearly shown (Barlass *et al.*, 1982) that GFLV-particles are present in very young meristematic tissue (i.e. meristematic domes of shoot apices) of grapevine material infected with fanleaf. The present study confirms that somatic embryogenesis is not successful in eliminating GFLV from grapevines - probably because: (1) NEPO viruses are not restricted to vascular tissue (Reynolds & Corbett, 1980) and are translocated to proliferating callus and embryoids and (2) GFLV is pollen transmitted (Hewitt *et al.*, 1970).

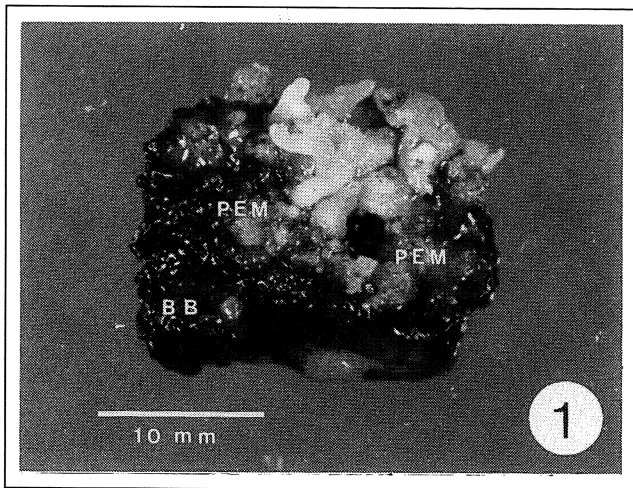


FIGURE 1

Pro-embryogenic masses (PEMs) developing out of nodular callus. Initially formed nodular callus turned brown/black (BB).

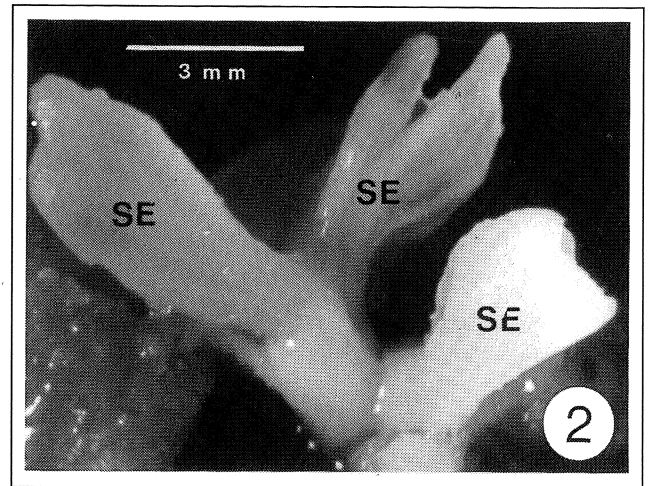


FIGURE 2

Heart/torpedo-shaped somatic embryos (SE) with cotyledons developing out of PEM.

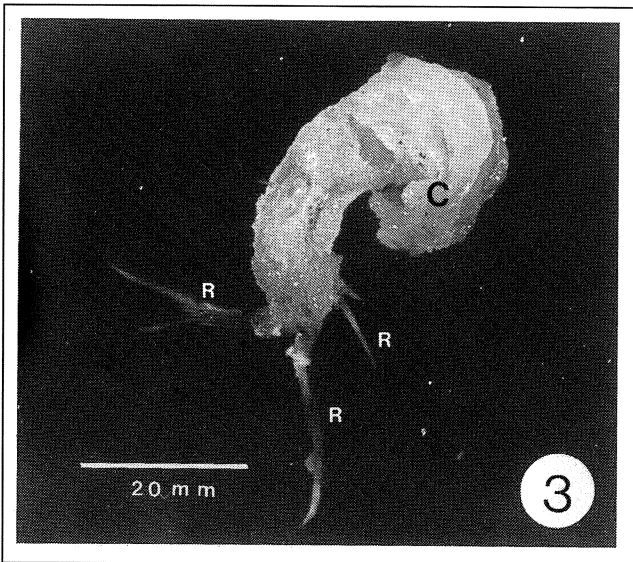


FIGURE 3

Mature somatic embryo, showing cotyledon (C) and root (R) development.

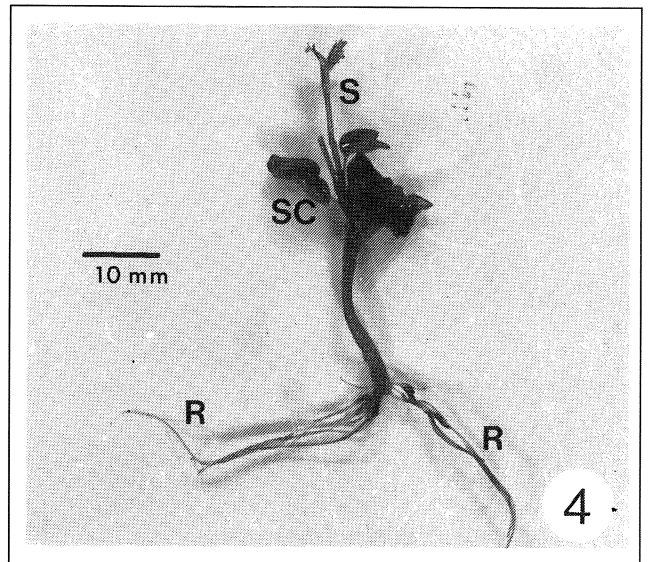


FIGURE 4

Germinated somatic embryo, showing shoot elongation (S), shrivelled cotyledons (SC) and roots (R).



FIGURE 5

Plants generated by somatic embryogenesis are vigorously growing in the growth chamber.

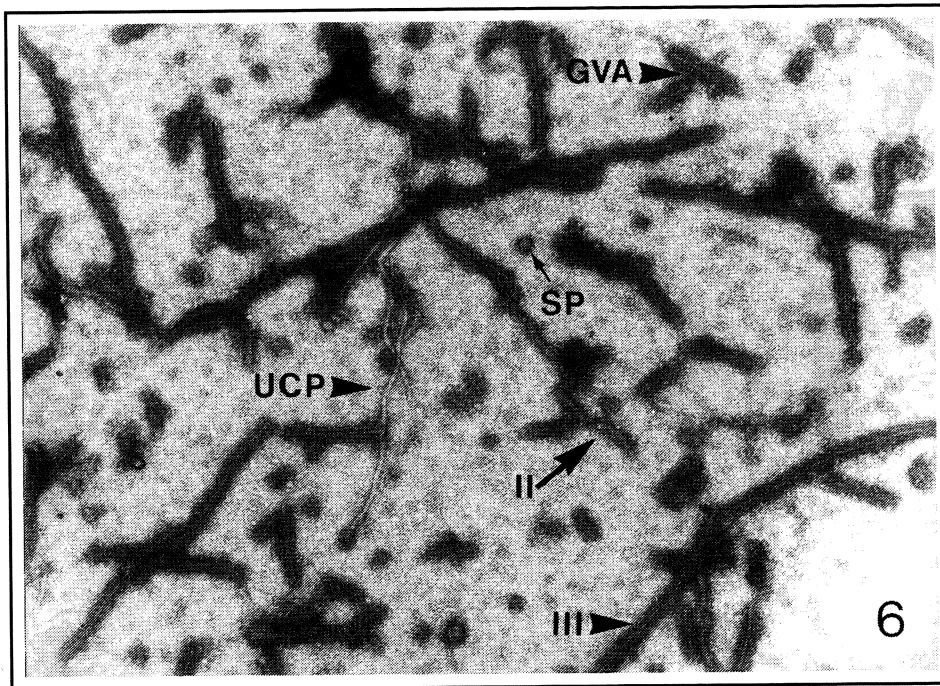


FIGURE 6

Electron micrograph of GVA, grapevine leafroll associated viruses II and III, an unidentified spherical virus-like particle (SP) and an undecorated closterovirus particle (UCP) present in extracts from source material of Roobernet, negatively stained with 2% uranyl acetate (130000 X).

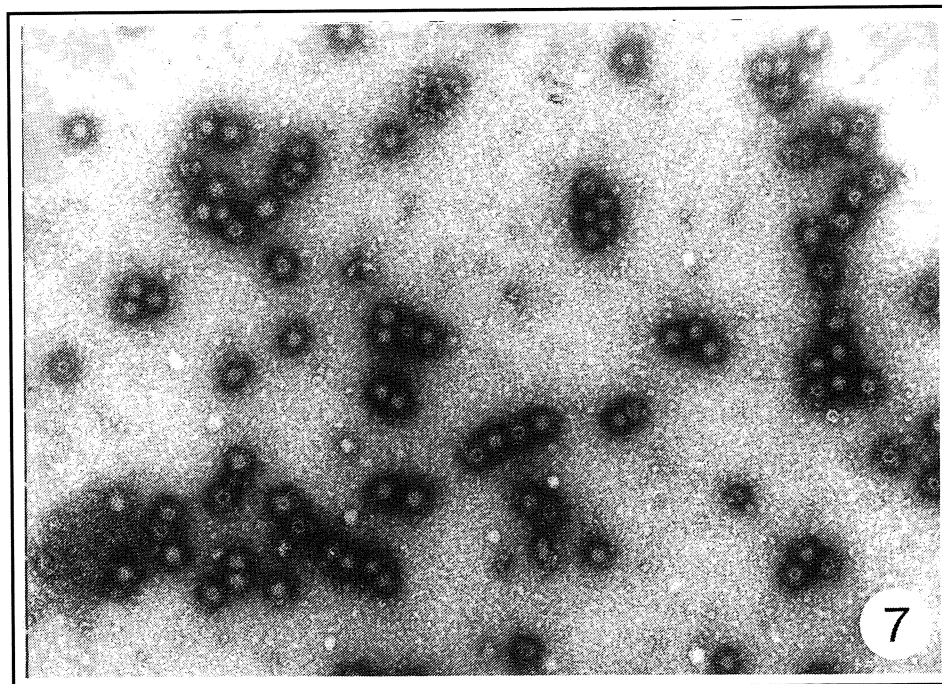


FIGURE 7

Electron micrograph of GFLV-particles present in source material of Rupestris du Lot, negatively stained with 2% uranyl acetate (171000 X).

TABLE 1

Indexing results of source material and regenerated somatic embryos and plantlets of the cultivars Roobernet and Rup. du Lot with ISEM and ELISA.

Virus Type	Source material **				Somatic embryos and plantlets ***			
	Roobernet		Rup. du Lot		Roobernet		Rup. du Lot	
	ISEM	ELISA	ISEM	ELISA	ISEM	ELISA	ISEM	ELISA
GVA	+	-			-	-		
GLRaV I	-	-			-	-		
GLRaV II	+	+			-	-		
GLRaV III	+	+			-	-		
Spherical particle	+	*			-	*		
GFLV			+	+			+	+

GVA = Grapevine virus A.

GLRaV = Grapevine leafroll associated virus.

GFLV = Grapevine fanleaf virus.

* ELISA kit to test for this virus was not available at time of indexing.

** Tests were performed on material sampled from one source vine.

*** In each cultivar tests were performed on five somatic embryos and five plantlets selected at random.

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