Phytopathol. Mediterr. (2008) 47, 115–121

In vitro thermotherapy and shoot-tip culture to eliminate *Caper latent virus* in *Capparis spinosa*

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Summary. *Capparis spinosa* plants in the Sicilian Islands, Italy, are frequently infected with *Caper latent virus* (CapLV). CapLV affects the vigour and longevity of caper plants and exacerbates the effect of other adverse biotic and abiotic factors. To have CapLV-free cuttings of caper for multiplication and new planting is therefore advantageous for a revival of this crop wherever it is cultivated. An improved procedure for the *in vitro* thermotherapy combined with the culture of the shoot tips of *C. spinosa* has been applied to caper material collected from Salina (Aeolian Archipelago) and Pantelleria. More than of 60% (Salina) and 90% (Pantelleria) of the shoot tips survived, and 89–93% of the regenerated plantlets were CapLV-free when tested by RT-PCR. This is the first report on eliminating CapLV from caper plants.

Key words: caper, meristem tip culture, virus–free plants.

Introduction

Having virus-free planting material is a prerequisite for healthy plants of good quality in all crops. Viral diseases are mainly problematic in vegetatively propagated plants, where the spatial and temporal spread of the virus is inevitable (Thresh, 2006). Tissue culture techniques such as meristem tip culture and thermotherapy, either alone or in combination (Nyland and Goheen, 1969; Walkey and Cooper, 1995; Faccioli and Marani, 1998; Barba *et al*., 2003) have been used on woody plant species to eliminate viruses and to provide virus-free germplasm for propagation (Spiegel *et al*., 1995; Karesova *et al*., 2002; Manganaris *et al*., 2003).

Capparis spinosa L. is a perennial shrub (Porter, 1967) grown commercially for its unopened flower buds, the capers, that are mainly used for culinary purposes as a spice. Caper plants grow naturally in the Mediterranean Basin, where Morocco and Turkey are the leading producers and exporters, harvesting capers from wild plantations. Since the 1970s, Italy has increased its caper industry through specialized cultivations in the Sicilian Islands, mainly Pantelleria and Salina (Aeolian Archipelago) but now production has drastically declined and for this reason designated for a specific market niche only. Sicilian growers usually expect a caper plantation to remain productive and profitable for 10 to 20 years; but at the moment, any newly established fields rapidly decrease in vigor and productivity. A number of factors have contributed to this decline. First, no suitable agronomic practices have been devised to ensure renewable and profitable diversification for caper crops since they are considered a feral species. Then, pathogens and pests have significantly reduced both quality and yield (Infantino *et al*., 2006). Of pathogens on capers, surveys have ascertained that three viruses are spread in the

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Sicilian Islands: *Caper latent virus* (CapLV), *Eggplant mottle dwarf virus* (EMDV) and *Cucumber mosaic virus* (CMV) (Tomassoli *et al*., 2006).

Caper latent virus is a member of the genus *Carlavirus* (Gallitelli and Di Franco, 1987; Brunt *et al*., 2000). The viral genome (~ 10.000 nt) consists of one positive single-stranded RNA molecule encoding the virus coat protein (CP) of 35 kDA. CapLV naturally infects caper plants without producing visible symptoms in growth, leaves, buds or fruits. However, latent infection obviously weakens the plants, making them more susceptible to abiotic and biotic stresses, that favour a rapid decline. CapLV is easily transmitted by vegetative propagation, so that it spreads from infected mother plants to cuttings used to establish new plantations.

The aim of this study was to find a way to eliminate CapLV and produce CapLV-free plants from caper germplasm different to that found in Salina and Pantelleria. This was a necessary precaution since the virus is so widespread in these islands, and since it occurs in all the interesting germplasm that have been genetically selected by genetists and agronomists (Sottile *et al*., 2008). As a result, an *in vitro* procedure to eradicate CapLV in the shoots was developed. This study is the first attempt to support mother stocks of healthy caper germplasm for nurseries.

Materials and methods

Plant materials

One caper genotype growing in Pantelleria and one in Salina, both naturally infected with CapLV, were used as a source of explants for raising *in vitro* infected stock cultures. The mother plants were grown in pots in a greenhouse and the leaves were examined by RT-PCR to ascertain the virus in the initial plant material.

Shoot culture

Green shoots (10 mm) with apical, or one or two nodal, buds were excised from the young branches of the potted mother plants. The shoots were surface-sterilized by soaking for 1 hour, washing in running water, and dipping them in 70% ethanol for 1 min and in 12% sodium hypochlorite for 25 min. Nodal segments were then thoroughly washed several times with sterilized distilled water. The sterilized shoots were placed in bijou plastic tubes (Fig. 1) containing Murashige and Skoog (1962) medium (MS1) amended with 8 g l^{-1} agar, 30 g l^{-1} sucrose, and growth regulators such as 3-indolebutyric acid (IBA 0.1 mg l^{-1}), 6-benzylaminopurine (BAP 0.5 mg l^{-1}) and 2-isopentenyladenine (2iP 0.1 mg l^{-1}). The MS1 medium was finally adjusted to pH 7.0. The cultures were maintained at a temperature of 23±1°C, relative humidity 70% with a 16-h day and an irradiance of 3500 Lux provided by cool white fluorescent lighting. After 30 days, single shoots were transferred to a multiplication medium (MS2) consisting of sucrose 30 g; IBA 0.001 mg l^{-1}, BAP 0.25 mg l^{-1}, GA 30.1 mg l^{-1} and agar 8 g l^{-1}. One set of shoots was produced as the initial stock for each genotype.

Fig. 1. Sterilized green caper shoot in a bijou plastic tube.

Thermotherapy and meristem culture

Two trials were carried out, the first on 150 well-developed shoots per genotype, the second on 130 shoots per genotype (Table 1). The tubes containing the shoots were incubated at 38±1°C with a 16-h day followed by an 8-h dark period in a growth chamber. In each test, the thermotherapy lasted from 45 days to a maximum of 60 days until a suitable number of well-preserved shoots had been collected. Meristems of 0.3–0.4 mm were then excised and cultured on MS1 medium. After three weeks, the meristem tips were transferred to solid MS2 for plant regeneration. Homogeneous explants (~3 cm long) were rooted by culturing for 30 days in a MS rooting medium with sucrose 30 g l^{-1}, agar 6 g l^{-1} and IBA 5 mg l^{-1}. The explants were acclimatized in plastic pots containing a mixture of garden soil, sand and zeolite in the ratio of 1:1:1 in a growth chamber as above. High humidity was ensured by covering the plantlets with clean perforated transparent polythene bags. The bags were gradually removed and 3–4 weeks later the plantlets were potted and transferred to the glasshouse.

Virus detection

After thermotherapy and shoot tips cultures, the occurrence of CapLV in the clones was assessed by RT-PCR. Leaf material (50 mg) from each *in vitro* plantlet was collected, immediately frozen, homogenized in liquid nitrogen and its total RNA extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). A one-step single-tube RT-PCR was performed as described in Tomassoli and Tiberini, 2006. Briefly, a set of specific primers (CapLV-730D: 5'- TGCATGATCAGGTGTCCGAGC-3'; CapLV-206R: 5'CCATCGTGTTGAAGAGGAATGTACT-3') was used in a reaction mix (25 μl) containing 2 μl of total RNA, 1.5 mM of MgCl, 2.5 μM of each dNTP, 0.4 μM of each primer, 20 units of RNase inhibitor (Invitrogen Life Technologies, Carlsbad, CA, USA), 1.25 units of AMV (Promega Corp. Madison, WI, USA) and 0.75 units of Taq (Promega). RT incubation was at 42°C for 60 min and was followed by a denaturation step at 95°C for 5 min and by 35 cycles of amplification reaction of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C and a final extension of the amplification products for 10 min at 72°C. The expected size of the amplification product was 730 bp. All explants were tested at least twice with this protocol.

Results

The survival rates of the heat-treated explants differed between the Pantelleria and the Salina genotypes in both trials. Nearly all (97%) the Pantelleria explants survived heat therapy, but the Salina explants showed signs of stress and had a lower survival rate (data not shown). Consequently, a greater number of Salina shoots were selected 45 days after heat treatment and only 15 well-preserved shoots were collected 60 days later for each trial (Table 1). Apex meristems (0.3–0.4 mm) were excised from 80 explants per genotype in the first trial and from 70 explants per genotype in the second trial and the shoot tips were regenerated *in vitro* (Fig. 2). The heat-sensitive behaviour of Salina germplasm resulted in a lower rate (67 and 60%) of survival and regrowth of shoot tips compared with Pantelleria germplasm (>90%) (Table 1). The RT-PCR assay was carried out 4 weeks and 8 weeks after the entire sanitation cycle (thermotherapy plus meristem tip culture) in samples collected from apex-derived plantlets cultured *in vitro* (Fig. 3). Based on the RT-PCR assays, a high percentage (>90%) of regenerated explants were found free from CapLV infection and there were no significant differences in virus eradication when results from different heat treatment periods and different caper genotypes were compared (Table 2). About 60% of CapLV-free explants rooted *in vitro* (Fig. 4), and were transferred to the glasshouse where more than 50% of plants still survived after two months. A final RT-PCR analysis of *in vivo* plantlets confirmed the assays.

Discussion

Thermotherapy and meristem culture are routine techniques for virus eradication but results vary depending on the plant material and the type of virus that is to be eradicated. Members of the *Carlavirus* genus have been treated successfully *in vitro* each in its own perennial host, such as *Chrysanthemum virus B* in *Chrysanthemum* spp. (Hakkaart *et al.*, 1964; Tomassoli *et al.*, 2005), *Hot latent virus* and *Hot mosaic virus* in *Humulus lupulus* (Kremheller *et al.*, 1989) and in other herbaceous species (Woo *et al.*,

Table 1. Regeneration of caper explants after *in vitro* thermotherapy combined with meristem tip culture.

Trial	Genotype	No. heat treated explants	No. cultured meristem tips		No. regenerated explants			
			45 days	60 days	45 days	%	60 days	%
A	Pantelleria	130	50	30	45	90	27	90
	Salina	130	65	15	55	85	10	67
B	Pantelleria	150	40	30	36	90	28	93
	Salina	150	55	15	46	84	9	60

Table 2. CapLV elimination in regenerated caper explants as determined by RT- PCR.

Trial	Genotype	No. explants tested		No. virus–free explants			
		45 days	60 days	45 days	%	60 days	%
A	Pantelleria	45	27	40	89	25	92
	Salina	55	10	50	91	9	90
B	Pantelleria	36	28	34	94	26	93
	Salina	46	9	43	93	8	89

2004). The results of the present study demonstrate that thermotherapy combined with meristem culture is also effective in producing CapLV-free caper.

In vitro thermotherapy and shoot tip culture enhanced the elimination (*c.* 90%) of CapLV, a virus that in Italy occurs in all commercial caper plantations and infects plants throughout Pantelleria. Even if the Salina genotype was more susceptible to the longer period (60 days) of high temperature exposure, the survival rates implied that the caper genotypes, both of which are native to the semi-arid zone, were suitable for the thermotherapy procedure. Results of the complete *in vitro* process were less encouraging, as CapLV-free *in vivo* caper plants represented only *c.* 34% of the treated, regenerated and acclimatized shoots.

In vitro propagation studies on caper were first reported in 1985 (Ancora and Cuozzo, 1985), using materials from Apulia, and there have been some studies since then to improve the seed germinability and the rooting of cuttings of different caper ecotypes and to achieve the rapid and large-scale multiplica-

Fig. 2. Shoot tips (0.3–0.4 mm) after heat treatment for *in vitro* plantlet regeneration.

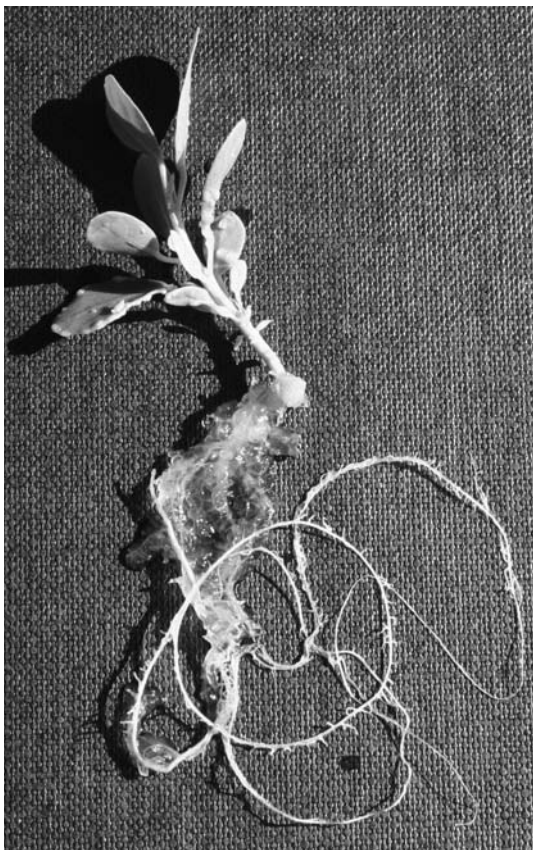

Fig. 4. Rooting of CapLV-free explants.

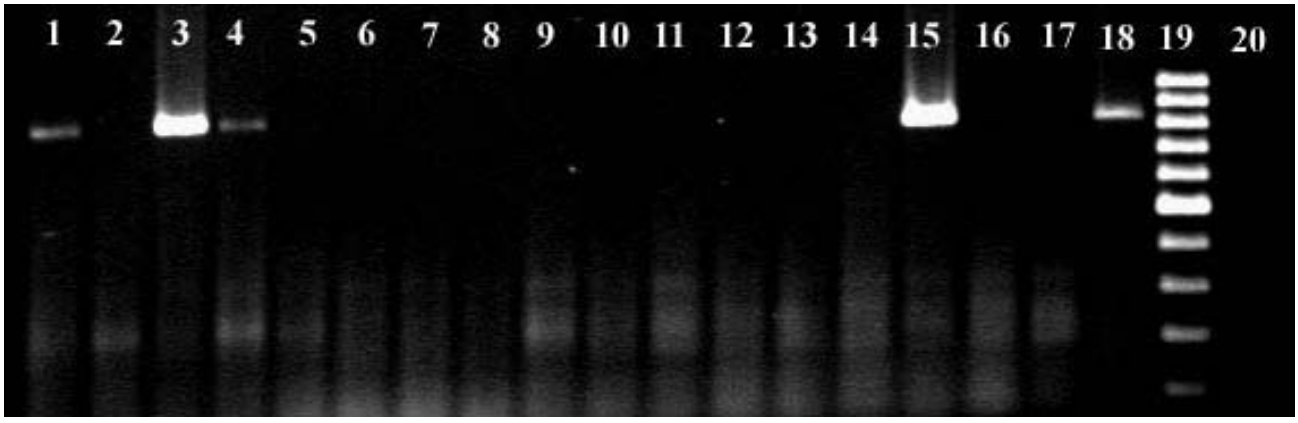

Fig. 3. Agarose gel electrophoretic analysis of the RT-PCR products (730 bp) of caper regenerated plantlets after thermotherapy and tip culture. Lane 1–10, Salina genotype; lane 11–16 Pantelleria genotype; line 17, virus-free seed-borne caper (negative control); line 18, CapLV infected caper (positive control); lane 19, 100 bp DNA ladder; lane 20, water control.

tion of this species (Rodriguez *et al*., 1990; Yldrm and Bayram, 2001; Chalak *et al*., 2003; Caglar *et al*., 2005; Horshati and Jambor-Benezur, 2006). In those studies, the rooting of caper explants and the acclimatization of plantlets still remained critical steps in the *in vitro* propagation process.

The present study demonstrated the effectiveness of thermotherapy and tip culture. Some other outstanding problems, such as the excessive sensitivity of Salina germplasm to high temperature and the low number of CapLV-free plants ultimately produced *in vivo*, might be overcome by preparing a larger stock of initial shoots.

In the study, RT-PCR was an effective and reproducible method for screening the numerous clones treated through the sanitation process. It proved to be sensitive, detecting low virus concentrations in even a few micrograms of *in vitro* material.

Over the last decade, *Capparis spinosa* has been regarded as a highly versatile crop that can thrive in marginal lands, growing well in salty or low fertility soils, and controlling soil erosion (Le Houerou, 2006). It also produces high levels of antioxidant in the capers and berries for alimentary (Tesoriere *et al*., 2007) and for cosmetic and pharmacological purposes (Panico *et al*., 2004), and there is a potential market for its beautiful flowers. It also produces a strong spice that has been in culinary use since ancient times. All these matters have boosted interest in a more intensive caper propagation, including that in the nursery. The sanitation techniques investigated in this paper should now be applied in order to produce virus-free nuclear stocks of native germplasm and subsequently to grow healthy caper on a larger scale to strengthen the caper industry both in countries where this crop has traditionally been grown, and in new countries such as Australia (Trewartha and Trewartha, 2005) .

Acknowledgements

This research was supported by the project "Caratterizzazione, miglioramento genetico-sanitario e difesa del cappero delle isole minori della Sicilia" with funds from the Regional Agricultural Bureau of Sicilian Region (L. 499/99).

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Accepted for publication: July 17, 2008