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Efficiency of eradication of four viruses from garlic (*Allium sativum*) by meristem-tip culture

Martin Verbeek[1], Paul van Dijk[2] and Peter M. A. van Well[1]
[1] *College for Higher Agricultural Education, P.O. Box 90108, 5200 MA 's-Hertogenbosch, The Netherlands;*
[2] *DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, The Netherlands*

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Abstract

Mechanical inoculation tests and ELISA with sap from garlic plants used for sanitation by meristem-tip culture revealed four viruses, viz. garlic common latent virus (GCLV) (carlavirus), the garlic strains of leek yellow stripe virus (LYSV-G), onion yellow dwarf virus (OYDV-G) (aphid-borne potyviruses), and onion mite-borne latent virus (OMbLV-G) (taxonomically unassigned virus). The same tests performed on explants grown *in vitro* showed elimination efficiencies of 100% for LYSV-G, 92% for OYDV-G, 62% for GCLV, and less then 54% for OMbLV-G.

Meristem tips excised from garlic cloves and bulbils, 0.15–1.0 mm in size, were tested for regeneration and efficiency of virus elimination after transfer to Murashige and Skoog medium. Successful regeneration into plantlets was obtained with 71% of the meristems from cloves and 72% of those from bulbils, but virus elimination was easiest from cloves: 38% of all explants from cloves and 25% of those from bulbils were virus-free. The efficiency of elimination increased with increasing weight of the cloves, irrespective of the virus. Small tip size seemed to favour virus elimination, but sizes smaller than 0.4 mm led to increasing failure of regeneration.

Micropropagation was most successful when cytokinins were omitted from the medium and the garlic shoot was split. Multiplication factors of 3–6 were obtained.

Introduction

Worldwide, all plants and bulbs of garlic (*Allium sativum*) harbour complexes of viruses due to exclusive vegetative propagation [Bos, 1983; Walkey, 1990; Van Dijk, 1994], which cause yield reductions up to 50% [Quiot *et al.*, 1972; Havránek, 1974; Messiaen *et al.*, 1981]. Since Mori [1971] in Japan applied meristem-tip culture to free garlic from virus infections, virus-free garlic clones have been produced in many countries, e.g. France [Quiot *et al.*, 1972; Messiaen *et al.*, 1994], Czechoslovakia [Havránek, 1972], China [Wang and Huang, 1974; Peiwen *et al.*, 1994], Korea [Hee-Don and Moo-Ung, 1979], Italy [Marani and Bertaccini, 1980; Bertaccini *et al.*, 1986], Argentina [Nome *et al.*, 1981; Conci and Nome, 1991], New Zealand [Bhojwani *et al.*, 1982], Spain [Peña-Iglesias and Ayuso, 1982], Taiwan [Lin, 1985], the United Kingdom [Walkey *et al.*, 1987; Walkey and Antill, 1989], and Austria [Abo el-Naga *et al.*, 1989]. The percentage of virus-free plants obtained from meristem tips varies and can be raised substantially by heat treatment prior to tissue culture [Walkey *et al.*, 1987; Conci and Nome, 1991; Peiwen *et al.*, 1994]. Up to a recent past the identity of the various viruses present in garlic was uncertain, therefore the efficiency of elimination of each single virus could not be reliably studied and documented.

Following detection and identification of mite-borne flexuous viruses [Van Dijk *et al.*, 1991; Van Dijk and Van der Vlugt, 1994], aphid-borne potyviruses [Van Dijk, 1993a], and carlaviruses [Van Dijk, 1993b] of *Allium* at Wageningen, we have studied the elimination of these viruses from a garlic selection by meristem-tip culture. The aim of our experiments was to compare the rate of elimination of each of the viruses from cloves and bulbils. The results also gave an insight in other factors that influence the efficiency of virus elimination. As a sideline, tentative experiments were done for improving micropropagation of garlic.

Materials and methods

Garlic parental material

Virus eradication was studied with a garlic selection obtained from the 'Stichting Roos & Lelie', Nijmegen, the Netherlands, indicated by them as 'Rocambole', originating from France and cultivated in the field in the Netherlands. Plants of this selection produce long inflorescences bearing large numbers of bulbils. Bulbs and bulbils from 26 plants used for meristem culture, referred to as 'parent plants', were kept separately. Two cloves and two bulbils from each of the parent plants were planted for virus screening in a greenhouse. The remaining cloves and bulbils were weighed and stored for four months in dry conditions at room temperature until excision of their meristems.

Micropropagation experiments were done with young shoots from cloves of various garlic accessions supplied by De Groot & Slot B.V., Heerhugowaard, the Netherlands.

Virus indexing

Plants grown from cloves or bulbils from parent plants, and plantlets or callus grown from meristem tips *in vitro*, were tested for virus infections by mechanical inoculation onto test plants and ELISA. Table 1 presents the criteria used to identify the currently recognised garlic viruses, and lists their acronyms and literature references.

Meristem-tip excision and culture

Cloves or bulbils used for meristem-tip excision were disinfected by immersion in a 70% (v/v) ethanol solution for approximately 10 s, then in 1.5% (w/v) sodium hypochlorite for 15 min, and twice in sterilised water for 5 min. Shoot tips consisting of the meristematic dome and 1–3 leaf primordia were excised with sterile tools under a laminar flow hood, and their size was recorded prior to transfer to flat-bottom glass tubes containing 10 ml of slanted Murashige and Skoog [1962] mineral salt medium with vitamins, 3% sucrose and 0.7% agar, pH 6.2 (measured before autoclaving). The tubes were placed in a growth chamber at 25 °C with a photoperiod of 16 h at 1,500 lux. When the plantlets were large enough for subculturing, they were transferred every three weeks to fresh medium and at each transfer, the leaves were removed. Occasional transfers after five weeks resulted in growth retardation, which was overcome by supplementing the medium with 0.5 mg l^{-1} naphtaleneacetic acid (NAA) and 1 mg l^{-1} 6-benzyladenine (BA). Removed leaves, or callus, were used for assessing the health status by inoculation onto test plants, ELISA, or electron microscopy.

In micropropagation experiments, cytokinins (kinetin, BA or 2-isopentenyladenine (2-i-P) at 2.5 mg ml^{-1}) were added to the medium to stimulate axillary shoot formation. Alternatively, no cytokinins were used but the shoots were cut lengthwise in two, before transferring them to fresh medium.

Statistical analysis and calculations

The results were statistically worked out using GENTSTAT 5 multiple regression analysis [Payne *et al.*, 1987].

Averages of combined classes were calculated as weighed averages (the sum of the products of figure and number of each class, divided by the sum of the numbers of all classes).

Table 1. Identification of garlic viruses

Virus group Virus (acronym)	Test plants and reactions or serological method	References
Carlavirus		
Garlic common latent virus (GCLV)	*Celosia argentea* var. *plumosa* 'Geisha': chlorotic local lesions later becoming necrotic or green rings; no systemic reaction *Chenopodium amaranticolor*: idem *C. murale*: idem *C. quinoa*: idem *Nicotiana occidentalis* accession P1: no local lesions: systemic necrosis may appear *Vicia faba*: no reaction	Van Dijk, 1993b
Shallot latent virus – garlic strain (SLV-G)	*Celosia argentea* var. *plumosa* 'Geisha': necrotic local lesions; no systemic reaction *Chenopodium amaranticolor*: idem *C. murale*: necrotic local lesions may appear; no systemic reaction *C. quinoa*: necrotic local lesions; no systemic reaction *Nicotiana occidentalis* accession P1: necrotic local lesions may appear; no system reaction *Vicia faba*: necrotic local lesions; usually no system reaction	Van Dijk, 1993b
Mite-borne flexuous virus		
Onion mite-borne latent virus – garlic strain (OMbLV-G)	*Celosia argentea* var. *plumosa* 'Geisha': usually no reaction *Chenopodium amaranticolor*: idem *C. murale*: chlorotic local lesions with etching and necrosis or green rings; no systemic reaction *C. quinoa*: no reaction *Nicotiana occidentalis* accession P1: no reaction *Vicia faba*: no reaction	Van Dijk *et al.*, 1991
Potyvirus		
Leek yellow stripe virus – garlic strain (LYSV-G)	ELISA [1]	Van Dijk, 1993a
Onion yellow dwarf virus – garlic strain (OYDV-G)	ELISA [1]	Van Dijk, 1993a

[1] Antisera to LYSV and OYDV from IPO-DLO; simultaneously incubation of plant extract and enzyme conjugate, with the addition of milk-powder to reduce nonspecific reactions (for details see Van Dijk, 1993a).

Results

Regeneration of meristem tips

Successful regeneration was achieved with 71% of meristem tips from cloves and from 72% of those from bulbils (Table 2). Part of the tips, viz. 8% and 16% of the explants from cloves and bulbils, respectively, did not develop subsequently on the medium (Table 2) either because of injury during manipulation or of too small size. Growth failure was not caused by the medium because it occurred only when explants were 0.4 mm or less, and its rate increased with decreasing tip size. Other explants survived transfer to the tube but did not regenerate into plantlets either. These explants produced only callus, or developed normally at first but died after some time of culturing *in vitro*. Death of shoots occurred in 21% and 13% of the meristem tips from cloves and bulbils, respectively, and was not clearly associated with tip size (Table 2).

Viruses detected in parent plants

Reactions on indicator hosts showed the presence of GCLV and the absence of SLV-G in the offspring of each of the 26 parent plants. ELISA revealed the presence of OYDV-G in the progeny of all, and of LYSV-G in the progeny of one of the

Table 2. Efficiencies of regeneration and of virus elimination of meristem tips from cloves or bulbils of garlic in relation with meristem-tip size and weight of clove or bulbil

Cloves/Bulbils Division by Division class	Weight (g)[1]	Size (mm)[2]	Meristem excision and culture						Virus elimination[6]									
			No growth[3]		Shoot death[4]		Regeneration[5]		LYSV-G		OYDV-G		GCLV		OMbLV-G[7]		All viruses	
			RN	%	RN	%	RN	%	RN	%	RN	%	RN	%	RN	%	RN	%
Cloves																		
Meristem-tip size[8]																		
D1	3.4	0.23	3/10	**30**	2/10	**20**	5/10	**50**	–	–	5/5	**100**	5/5	**100**	4/5	**80**	4/5	**80**
D2	4.6	0.33	1/12	**8**	2/12	**17**	9/12	**75**	–	–	8/9	**89**	8/9	**89**	3/8	**38**	3/9	**33**
D3	5.4	0.50	0/16	**0**	3/16	**19**	13/16	**81**	1/1	**100**	14/14	**100**	8/14	**57**	6[10]/18	**75**	6/14	**43**
D4	4.5	0.80	0/14	**0**	4/14	**29**	10/14	**71**	–	–	9/11	**82**	4/11	**36**	2[10]/4	**50**	2/11	**18**
Weight of clove[9]																		
W1	2.7	0.48	3/18	**17**	2/18	**11**	13/18	**72**	–	–	12/13	**92**	7/13	**54**	2/7	**29**	2/13	**15**
W2	4.5	0.47	0/16	**0**	5/16	**31**	11/16	**69**	–	–	10/12	**83**	6/12	**50**	4/6	**67**	4/12	**33**
W3	6.5	0.53	1/18	**6**	4/18	**22**	13/18	**72**	1/1	**100**	14/14	**100**	12/14	**86**	9/12	**75**	9/14	**64**
Average	*4.6*	*0.49*	*4/52*	***8***	*11/52*	***21***	*37/52*	***71***	*1/1*	***100***	*36/39*	***92***	*25/39*	***64***	*15/25*	***60***	*15/39*	***38***
Bulbils																		
Meristem-tip size[8]																		
D1	1.2	0.21	3/15	**20**	1/15	**7**	11/15	**73**	–	–	10/11	**91**	7[10]/11	**64**	3/7	**43**	3/11	**27**
D2	1.1	0.33	2/9	**22**	0/9	**0**	7/9	**78**	1/1	**100**	7/7	**100**	5/7	**71**	2/5	**40**	2/7	**29**
D3	1.0	0.46	0/7	**0**	2/7	**29**	5/7	**71**	–	–	5/6	**83**	2/6	**33**	1/2	**50**	1/6	**17**
D4	1.5	0.70	0/1	**0**	1/1	**100**	0/1	**0**	–	–	–	–	–	–	–	–	–	–
Weight of bulbil[9]																		
W1	0.8	0.31	1/10	**10**	2/10	**20**	7/10	**70**	–	–	6/7	**86**	3/7	**43**	0/3	**0**	0/7	**0**
W2	1.2	0.30	3/12	**25**	1/12	**8**	8/12	**67**	1/1	**100**	8/9	**89**	5/9	**56**	4/5	**80**	4/9	**44**
W3	1.5	0.34	1/10	**10**	1/10	**10**	8/10	**80**	–	–	8/8	**100**	6/8	**75**	2/6	**33**	2/8	**25**
Average	*1.1*	*0.31*	*5/32*	***16***	*4/32*	***13***	*23/32*	***72***	*1/1*	***100***	*22/24*	***92***	*14/24*	***58***	*6/14*	***43***	*6/24*	***25***

[1] Average weight of cloves or bulbils used for meristem excision in the respective class.
[2] Average size of the meristems in the respective class.
[3] Figures represent the number of explants that failed to grow out of the number of explants incubated, as a relative number (RN) and as a percentage (%).
[4] Figures represent the number of cultured explants that died after initial growth, or produced callus only, out of the number of explants incubated, as a relative number (RN) and as a percentage (%)
[5] Figures represent the number of regenerated explants out of the number of explants incubated, as a relative number (RN) and as a percentage (%).
[6] Relative number and percentage of explants freed from the garlic strain of leek yellow stripe virus (LYSV-G), the garlic strain of onion yellow dwarf virus (OYDV-G), garlic common latent virus (GCLV), and the garlic strain of onion mite-borne latent virus (OMbLV-G), or all viruses (All viruses).
[7] Actual figures for OMbLV-G are lower than the figures presented because only plants free of GCLV could be tested for presence of OMbLV-G (see text).
[8] Classes of meristem-tip sizes for cloves and bulbils were as follows: D1 = from 0.15 till 0.3 mm; D2 = from 0.3 till 0.4 mm; D3 = from 0.4 till 0.6 mm; D4 = from 0.6 up to 1.0 mm.
[9] The mean weight of cloves and bulbils of each of the parent plants was recorded. Classes of weights of cloves and bulbils used for excision of meristem tips were as follows: W1-cloves = from 1.4 till 4.0 g; W2-cloves = from 4.0 till 5.0 g; W3 cloves = from 5.0 up to 10.0 g; W1-bulbils = from 0.6 till 0.9 g; W2-bulbils = from 0.9 till 1.4 g; W3-bulbils = from 1.4 up to 1.9 g.
[10] Virus infection of one of the plants was detected in the second test only.

parent plants. OMbLV-G, isolate As185, was transmitted from at least one of the parent plants by transfer of mites, present in large numbers on the leaves of the plants, to onion and leek [Van Dijk *et al.*, 1991]. This virus, for which no ELISA is available yet, could not be indexed by sap inoculation because its symptoms on *Chenopodium murale*, the only indicator for OMbLV-G, are similar to those of the ubiquitous GCLV. Symptoms of GCLV and OMbLV-G are presented in Fig. 1. We assumed that OMbLV-G was symptomlessly present in all parent plants since they were infested by large numbers of mites. Detection of OMbLV-G in several cultured explants by electron microscopy or on test plants (see below) supports this assumption.

Because differences in the rate of virus elimination between cloves and bulbils might be due to differences in virus concentration, multiple dilution extracts from such propagules were compared. However, concentrations of GCLV or OYDV-G in cloves and bulbils did not differ essentially (data not shown).

Virus infections in explants

The presence of viruses in plantlets or callus grown from meristem tips varied considerably (Table 2). Many of the plants were still infected with GCLV and/or OMbLV-G, but most were freed from OYDV-G. No plant was found infected by OYDV-G alone. LYSV-G was not found in either of the two plants obtained from the parent plant infected by this virus. In mixed infections of OMbLV-G and GCLV, OMbLV-G could not be distinguished from GCLV. Sap from *in vitro* plants, which gave reaction in *C. murale* and not in *C. quinoa* and *C. argentea* var. *plumosa* 'Geisha' (thus not infected by GCLV), contained the highly flexuous particles of OMbLV-G when examined in the electron microscope. Plants, scoring negative in inoculation tests and ELISA, proved to be virus-free when investigated by electron microscopy.

Tests repeated after no less than three weeks and up to six months confirmed the results for LYSV-G and OYDV-G, but showed one plant to contain GCLV and two plants to contain OMbLV-G not detected in the first test (Table 2, footnote 10).

Elimination rates of the four viruses

Each of the four viruses infecting the parental material could be removed by excision of the meristem tips, but their elimination rates differed. There was no essential difference in elimination efficiencies between cloves and bulbils (Table 2), and the average elimination efficiencies calculated from the figures in Table 2 were 100, 92, 62, and 54% for LYSV-G, OYDV-G, GCLV, and OMbLV-G, respectively. Although the successfully cultured meristems included only two from the parent plant containing LYSV-G, the removal of this virus from both meristems indicates that the efficiency of eradication is of the same high order as that of the other potyvirus OYDV-G (92%). The figure of 54% for OMbLV-G cannot be compared with the figures of the other viruses without correction for the fact that only plants free from GCLV were tested for OMbLV-G. By comparison with the behaviour of other virus mixtures with respect to efficiency of elimination, it is assumed that removal of OMbLV-G was less successful from meristems still infected with GCLV than from those freed from GCLV. For example, our data showed that OYDV-G was present in 21% and 0% of the plants still infected by or freed from GCLV, respectively. We therefore conclude that OMbLV-G was removed from less than 54% of the explants. Thus the eradication of OMbLV-G, from either tips and bulbils, was more difficult than that of GCLV, and much more difficult than that of the potyviruses LYSV-G and OYDV-G (Table 2). OMbLV-G reemerged in two explants, GCLV in one explant, and OYDV-G in none of the explants when they were retested (Table 2, footnote 10). This shows that the more difficult it was to remove a virus from the meristem, the more often it remained present at an undetectably low level.

Size of excised meristem tip and efficiency of virus elimination

The size range of the meristem tips from 0.15 to 1.0 mm was divided into classes S1–S4 of increasing size, chosen in such a way that each class contained about an equal number of them. Virus elimination was thereafter calculated for each size class (Table 2). Comparison of these figures showed that, except for minor deviations,

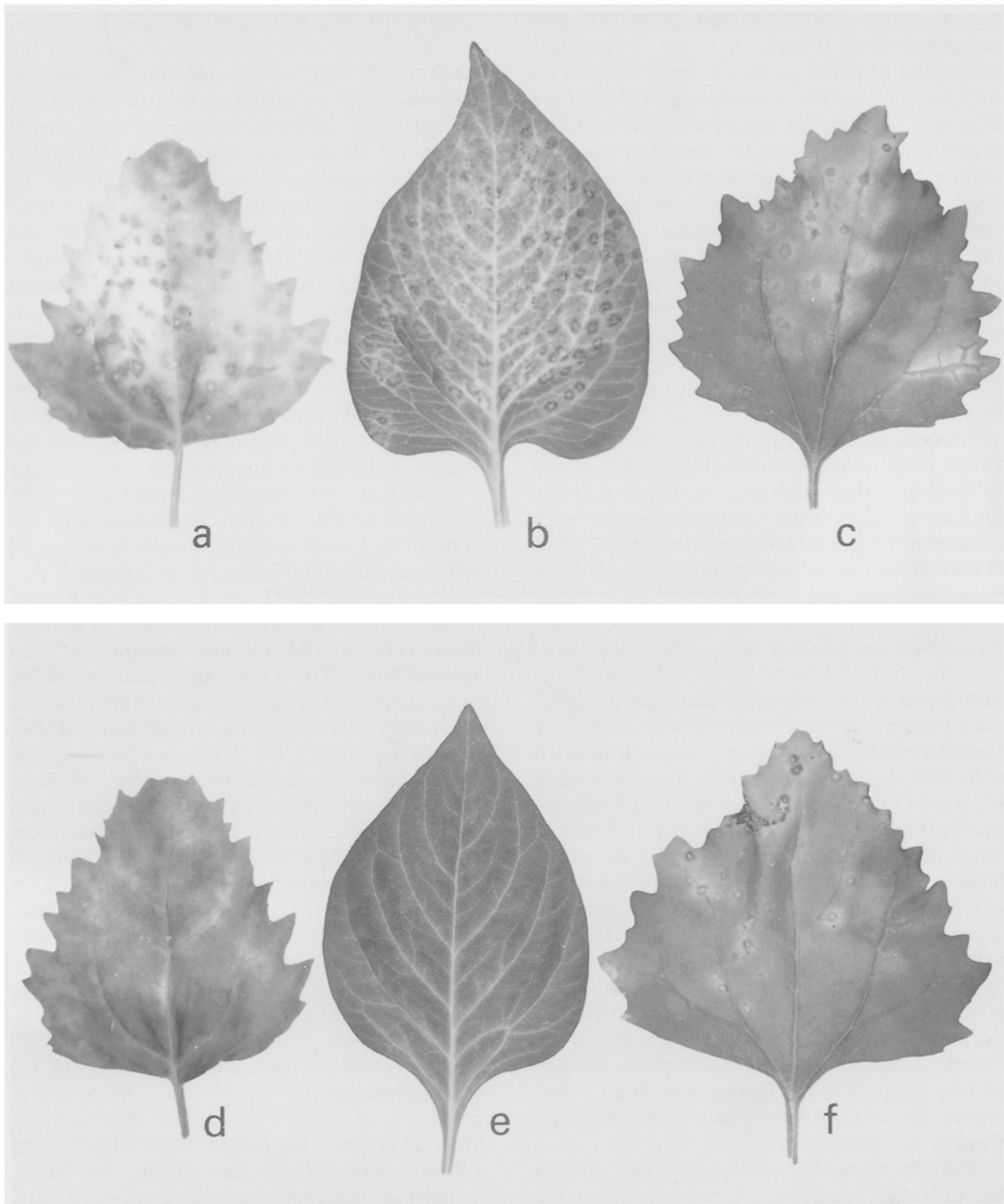

Fig. 1. Reactions of garlic common latent virus (GCLV) isolate As186I (a–c) and the garlic strain of onion mite-borne latent virus (OMbLV-G) isolate As185 (d–f) in leaves of *Chenopodium quinoa* (a, d), *Celosia argentea* var. *plumosa* 'Geisha' (b, e) and *Chenopodium murale* (c, f). Note the absence of local lesions in *C. quinoa* and *C. argentea* after inoculation with OMbLV-G (d, e) and the similarity of local lesions of GCLV and OMbLV-G in *C. murale* (c and f, respectively).

the efficiency of eradication increased with decreasing meristem-tip size. Figures for eradication of the whole complex of viruses ranged from 18% in S4 to 80% in S1 for meristems from cloves, and from 17% in S3 to 27% in S1 for meristems from bulbils. This tendency was also observed for each virus separately, although least for OYDV–G (Table 2). The effect of tip size on the rate of virus elimination was not statistically significant for cloves ($p = 0.15$) and bulbils ($p = 0.70$).

Efficiency of virus elimination from cloves and bulbils

Explants had a 38% chance to be virus-free when excised from cloves against 25% when excised from bulbils, due to the slightly easier elimination of GCLV and OMbLV-G from cloves (Table 2). Eradication figures of 46% and 25% were calculated for meristem-tips of size classes S1 to S3 for cloves and bulbils, respectively (Table 2). Meristem-tip sizes for the numbers in classes S1 to S3 averaged 0.40 mm and 0.31 mm for cloves and bulbils, respectively. Thus, virus eradication from bulbils was more difficult than from cloves, despite the relatively small average size of the tips from bulbils. These differences seem not to be due to differences in virus concentration (data not shown), but may be due to the relatively small size of the meristems in bulbils, which complicates meristem excision.

Weight of clove or bulbil and efficiency of virus elimination

When the weights of cloves or bulbils (averaged per parent plant) were grouped into three classes, it appeared that virus elimination had been much more successful with increasing weight of the cloves from which the meristems were excised. An increase of average clove weight from 2.7 to 6.5 g raised the relative number of virus-free explants from 15 to 64% (Table 2). This tendency was statistically highly significant ($p = 0.02$). It could not be explained by differences in meristem-tip diameter, since average tip diameters varied little among the three weight classes (Table 2). Figures for bulbils did not resemble those of cloves (Table 2), and the differences between weight classes of bulbils were not statistically significant ($p = 0.50$). This might be explained by the fact that the weight of the bulbils of one and the same plant varied largely and only average weights were recorded in these experiments.

Micropropagation

In vitro multiplication of garlic plantlets was usually poor with all cytokinins tested. Especially with BA, abnormal, vitrified plants were obtained. Much improvement was achieved by omitting cytokinins from the medium, combined with splitting the garlic shoots in half. Multiplication factors from 3 to 6 were recorded, depending on the cultivar. Subculture lasted approximately 5 weeks. The plants could successfully be rooted and transferred to soil.

Discussion

Production of virus-free garlic stock by meristem-tip culture has begun in a number of countries. Rates of success in culturing meristem tips from garlic cloves of 68% [Abo el-Naga *et al.*, 1989] and 78% [Conci and Nome, 1991] have been reported. Percentages of virus-freedom of explants from garlic cloves were 14% in Argentina [Conci and Nome, 1991], 25–50% in England [Walkey *et al.*, 1987], and 50% in Austria [Abo el-Naga *et al.*, 1989]. The average percentages obtained in our work (71% regeneration and 38% virus-freedom) are well within this range. Screening of explants for virus-freedom remains problematic with the continuing paucity of information on viruses infecting garlic, confusion about their identity, and ways of reliable and rapid detection. Walkey *et al.* [1987] and Conci and Nome [1991] partially identified the viruses in garlic clones submitted to meristem culture, but they did not compare their rates of elimination. Abo el-Naga *et al.* [1989] mentioned that 81% of the plantlets from meristem culture were freed from 'garlic latent virus', now named GCLV [Van Dijk, 1993b]. We were now able to identify four viruses in a French garlic selection as viruses also generally infecting garlic in other parts of the world [Van Dijk, 1994], and to compare their elimination rates. The economically most important

aphid-borne potyviruses LYSV-G and OYDV-G [Van Dijk, 1993a] were eliminated most efficiently, whereas the mite-borne flexuous virus OMbLV-G was eliminated least efficiently, and the aphid-borne carlavirus GCLV was intermediate. This is in line with the notion that some viruses are more readily eliminated than others from the same host [Stone, 1968; Quak, 1977; Mellor and Stace-Smith, 1977].

Virus-free garlic stocks may be readily reinfected by OMbLV-G due to widespread occurrence, large population densities, very small dimensions and easy dissemination of its vector *Aceria tulipae* (the dry bulb mite), and omnipresence of the virus in garlic [Van Dijk *et al.*, 1991].

The efficiency of virus elimination in our experiments increased with decrease of meristem-tip size, but sizes smaller than 0.4 mm resulted in a growth failure of up to 30% (Table 2). Similar effects of tip size on virus elimination and regeneration were reported for garlic [Quiot *et al.*, 1972; Peiwen *et al.*, 1994], carnation [Stone, 1963], and potato [Mellor and Stace-Smith, 1977].

In average, virus elimination was less efficient from bulbils than from cloves, possibly because meristems of bulbils are comparatively smaller. Virus elimination was strongly favoured by high weight of the cloves that produce the meristems (Table 2). We are not aware of any other publication reporting this relationship. The relatively large dimensions of the organs in heavy cloves, facilitating the removal of more tissue of leaf primordia from around the meristem, may at least partially explain this effect. The larger bulbs should therefore be selected for meristem culture. Such bulbs result from the most vigorous plants, the selection of which has been recommended for the production of virus-free propagation material since they may carry resistance or tolerance to harmful potyviruses [Van Dijk and Sutarya, 1992; Van Dijk, 1993a].

The fact that no cytokinins were required in micropropagation of garlic when cutting the plantlet into two, might be explained by the possible destruction of the apex, thus decreasing apical dominance.

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