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CRYOPRESERVATION OF APICES ISOLATED FROM GARLIC (*Allium sativum* L.) BULBILS AND CLOVES

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Summary: This paper reports on the successful application of the vitrification technique for cryopreserving apices sampled from bulbils and cloves of garlic. With bulbils, survival and regrowth of control apices were generally high after treatment with PVS2 and PVS3 vitrification solutions. However, after cryopreservation, apices treated with PVS3 solution had a much higher regrowth rate than those treated with PVS2 solution. An experiment performed with a total of 11 garlic accessions showed that the success of cryopreservation depended on the size of the bulbils from which apices had been dissected. The following trend was observed as regards regorwth of cryopreserved apices: the highest regrowth frequency was achieved with apices taken from large bulbils, whereas no regrowth was noted with apices from small bulbils, and regrowth of apices from medium-size bulbils was intermediate. Apices from cloves had higher survival and regrowth frequencies than those from bulbils.

Keywords: garlic; *Allium sativum* L.; apices; bulbils; cloves; cryopreservation; vitrification.

Introduction

Garlic (*Allium sativum* L.) is a crop of increasing interest, not only because of its use as spice in many countries but also because of its importance as a source of useful biochemical compounds such as alliin which makes this species one of the most important medicinal plants in the world (2, 8, 14, 18). Therefore, over recent years, genebank curators and breeders have considerably increased the number of garlic accessions in their collections. Since garlic is so far seed-sterile, genetic resources of this species are traditionally maintained as whole plants in field collections. Field genebank conservation presents certain drawbacks that limit its efficiency and threaten its security (20). Cryopreservation of apices represents the only current option for the safe long-term conservation of vegetatively propagated crops. Apices can be cryopreserved using classical two-step freezing, or various new protocols including encapsulation-dehydration, vitrification, encapsulation-vitrification and droplet freezing (3, 4). For numerous species, the new protocols, which are based on physical and/or osmotic dehydration of samples followed by vitrification of internal solutes during rapid cooling, thus avoiding detrimental ice formation give better results than classical, two-step freezing protocols which are based on freeze-induced dehydration (3, 19).

Two papers only report the successful cryopreservation of garlic using vitrification with apices sampled from cloves (5, 13). In both reports, the apices were cryopreserved using the vitrification technique. However, no information is available on the potential use of apices sampled from bulbils for cryopreservation.

In garlic, like in other bulbous crops, apices can be found in limited number only in the basal plate of the bulbs (cloves). The dissection of apices is therefore more difficult than with many herbaceous species. Apices are also found in bulbils which are formed in the inflorescences of the majority of garlic accessions. Bulbils present also the advantage that they are not in direct contact with the soil and are, therefore, less infested with soil-borne micro-organisms than cloves. Usually, flower heads of garlic contain a large number of bulbils, which are vegetative propagules. They are thus clonal and provide a much larger amount of genetically and physiologically homogenous material than cloves.

In the present work, a cryopreservation protocol was developed for apices sampled from bulbils of a total of eleven garlic accessions originating from the Polish and German garlic germplasm collections. Apices sampled from cloves of several accessions were also cryopreserved to allow comparison between the two types of materials.

Material and Methods

Plant Material

Bulbils of a total of eleven clones were used, originating from the IPK genebank in Gatersleben, Germany (All- and TAX- numbers), and the RIVC genebank in Skierniewice, Poland (S- and N- numbers). The material was grouped according to the size of the bulbils, which is one of the characteristic features of an accession recorded in genebanks (6, 7):

- Large bulbils (370-420 mg): All 290, All 499, All 844;
- Medium-size bulbils (100-160 mg): All 111, All 232, All 815;
- Small bulbils (40-80 mg): 31 S, 32 S, 73 N, All 1165, TAX 1125.

Apices sampled on cloves from accessions All 232, All 290, All 499, and All 815 were also used for some cryopreservation experiments.

Explant preparation

Before explant isolation, bulbs and bulbils were stored at 10°C from the harvest time in August 1997 until explant isolation (first experiment in October 1997, the last one in June 1998). After removal of the dry outer scales, bulbils and cloves were sterilized by placing them in 70% ethanol for 2 min, then in a 3% sodium hypochlorite solution for 15 min. Explants of approximately 1 mm in diameter and 3 mm in length, containing the meristem, the surrounding leaf primordia and a basal part were dissected from bulbils and cloves. Preliminary experiments (data not shown) had demonstrated that this was the optimal explant size.

Cryopreservation procedure

After isolation, apices were cultivated overnight on stantard medium (semi-solid MS medium (11) + 0.1 mg/L indole acetic acid and 0.1 mg/L kinetin (10) with 3% sucrose) at 25°C in the dark. The explants were immersed in a loading solution containing 2M glycerol and 0.4M sucrose for 20 min at room temperature. Apices were then transferred in 2ml polypropylene sterile cryotubes and treated for 0 to 300 min at room temperature with the vitrification solutions PVS2 (30% glycerol + 15% ethylene glycol + 15 % (w/v) dimethyl sulfoxide in liquid culture medium; 17) or PVS3 (50% sucrose + 50% glycerol in liquid culture medium;12).

After pretreatment, the cryotubes with the apices suspended in 0.5 ml of vitrification solution were plunged into liquid nitrogen (LN) where they were kept for at least 1 h. Rapid rewarming was achieved by plunging the samples in a 40°C water-bath until the cryoprotectant solution became liquid. Explants were then rinsed with standard liquid medium containing 1.2 M sucrose for 10 min, and placed on standard solid medium containing 0.3 M sucrose in the dark for 1 d. Apices were then transferred on standard medium with 3% sucrose and cultivated under a photoperiod of 16h light/8 h dark with a light intensity of 60-80 $\mu mol.cm^{-2}.s^{-1}$ at 25°C.

Assessment of survival and regrowth

Two characteristic stages were defined to evaluate the results: survival was observed 14 d after cryopreservation by counting the number of apices showing greening and swelling. Regrowth of apices was evaluated one month after cryopreservation by counting the number of apices which had produced new leaves. Developing plantlets were transferred onto MS medium with 0.1 mg/L NAA and 0.5 mg/L 2iP (1) for further growth and multiplication.

In all experiments performed with apices sampled on bulbils, a minimum of 20 apices were used per experimental condition. However, in experiments with apices sampled on cloves, 5 apices only were used per experimental condition. All differences reported in survival and regrowth rates of apices were evaluated by χ^2 tests using the Yates' correction factor for small samples.

Results

Survival of control apices was high (comprised between 50 and 100%) with both vitrification solutions, whatever the treatment duration employed (Table 1). After cryopreservation, a drop in survival rate of apices was observed with both vitrification solutions, but it was more accentuated after treatment with PVS2 solution and varied depending on the accession. The survival of apices cryopreserved after treatment with PVS3 solution was relatively high and comparable amongst the three accessions tested, from an average of 76% for All 232 to an average of 83% for All 844. By contrast, survival after treatment with PVS2 solution and cryopreservation was nil for apices of accession All 232 and was 17 and 37% on average for All 844 and All 815, respectively.

Table 1: Effect of vitrification solution employed (PVS2 or PVS3) and of treatment duration on the survival rate (%) of control (-LN) and cryopreserved (+LN) apices from bulbils of three garlic accessions. Twenty to 60 apices were employed per experimental condition. Data underlined are significantly different between treatments (p: 0.05).

		Survival (%)					
		All 232		All 815		All 844	
	Treatment duration (min)	PVS2	PVS3	PVS2	PVS3	PVS2	PVS3
-LN	0	100	100	100	100	100	100
	60	50	100	90	100	100	100
	120	100	100	75	100	100	100
	180	100	100	80	100	100	100
	240	100	87	65	100	80	100
	300	100	100	70	100	90	100
+LN	0	0	68	35	34	55	60
	60	0	77	80	80	15	83
	120	0	82	45	83	5	75
	180	0	85	40	98	15	95
	240	0	73	10	95	0	90
	300	0	68	10	95	10	93

Regrowth of control apices was generally high in most experimental conditions (Table 2). No or little differences were observed in the results obtained after treatment with PVS2 and PVS3, except with accession All 815 for which the longest treatment durations with PVS2 led to a drastic reduction in survival in comparison with PVS3.

After cryopreservation, no regrowth of apices was achieved after treatment with PVS2, except with accession All 815 without PVS2 treatment where 10% regrowth was achieved. When apices were cryopreserved after treatment with PVS3, regrowth could be achieved with the three accessions tested. The regrowth rate was very low with All 232 and All 815, and higher with All 844, for which a maximum of 60% regrowth was achieved after 240 min of treatment with PVS3.

Table 2: Effect of vitrification solution employed (PVS2 or PVS3) and of treatment duration on the regrowth rate (%) of control (-LN) and cryopreserved (+LN) apices from bulbils of three garlic accessions. Twenty to 60 apices were employed per experimental condition. Data underlined are significantly different between treatments (p: 0.05).

		Regrowth (%)					
		All 232		All 815		All 844	
	Treatment duration (min)	PVS2	PVS3	PVS2	PVS3	PVS2	PVS3
-LN	0	80	53	90	93	100	100
	60	40	43	75	76	100	90
	120	70	45	45	73	100	83
	180	55	48	30	78	90	83
	240	55	55	15	66	70	90
	300	55	53	10	51	65	70
+LN	0	0	0	10	0	0	0
	60	0	2	0	22	0	13
	120	0	10	0	27	0	53
	180	0	7	0	10	0	40
	240	0	17	0	10	0	60
	300	0	8	0	15	0	33

The survival rate of control apices was high (90-100%) with all 11 accessions tested, and in most cases no optimal duration of treatment with PVS3 solution could be determined (Table 3). After cryopreservation, survival was achieved with 10 out of the 11 accessions employed and was generally lower than that of control apices. Survival was higher with apices sampled on large and medium-size bulbils than on small ones. The optimal duration of treatment with PVS3 solution varied depending on the accession and was comprized between 60 and 240 min.

Table 3: Optimal duration of treatment with PVS3 vitrification solution and maximal survival rate (%) of control (-LN) and cryopreserved (+LN) apices of 11 garlic accessions with different bulbil sizes (L: large; M: medium; S: small). Ten to 60 apices were employed per experimental condition. (-: no optimal treatment duration determined).

		-LN		+LN	
Accession number	Type of bulbil	Treatment duration (min)	Survival (%)	Treatment duration (min)	Survival (%)
All 290	L	0-300	100	120-180	100
All 499	L	90	100	120	88
All 844	L	0-300	100	180	95
All 111	M	0-300	100	60	93
All 232	M	0-300	100	180	85
All 815	M	0-300	100	180	98
All 1165	S	0-20	90	-	0
TAX 1165	S	0-300	100	210	60
31 S	S	0-300	100	240	75
32 S	S	0-150	100	150	80
73 N	S	0-300	100	60	25

The regrowth rate of control apices was generally lower than their survival rate and varied between 15 and 100%, depending on the accession (Table 4). The optimal durations of treatment with PVS3 solution varied between 0 and 240 min. After cryopreservation, no regrowth was obtained with apices sampled on small bulbils. Regrowth rates of apices sampled on large bulbils were higher in all cases except one than those obtained with medium-size bulbils.

Table 4: Optimal duration of treatment with PVS3 vitrification solution and maximal regrowth rate (%) of control (-LN) and cryopreserved (+LN) apices of 11 garlic accessions with different bulbil sizes (L: large; M: medium; S: small). Ten to 60 apices were employed per experimental condition. (-: no optimal treatment duration determined).

		-LN		+LN	
Accession number	Type of bulbil	Treatment duration (min)	Regrowth (%)	Treatment duration (min)	Regrowth (%)
All 290	L	90	90	120	100
All 499	L	120-150	43	180	13
All 844	L	0	100	120	53
All 111	M	60	68	60-240	15
All 232	M	240	55	240	17
All 815	M	0	95	120	28
All 1165	S	0-20	90	-	0
TAX 1165	S	120	70	-	0
31 S	S	0	65	-	0
32 S	S	30	65	-	0
73 N	S	150	15	-	0

No significant differences were noted between the survival rates of apices from bulbils and cloves (Table 5). By contrast, regrowth rates of apices sampled from cloves were much higher than those obtained with apices sampled from bulbils. The average regrowth rates were 78 and 59% before cryopreservation and 42 and 13% after cryopreservation, for apices from cloves and bulbils, respectively.

Table 5: Effect of duration of treatment with PVS3 vitrification solution on the survival and regrowth rates (%) of control (-LN) and cryopreserved (+LN) apices sampled on bulbils and cloves of garlic. Four accessions (All 232; All 290; All 499; All 815) were employed. With bulbils, a total of 151 apices were employed per experimental condition (60 apices from All 232;10 from All 290; 40 from All 499; 41 from All 815). With cloves, 20 apices were used per experimental condition (5 apices from each accession).

	Survival (%)				Regrowth (%)			
	Bulbils		Cloves		Bulbils		Cloves	
Treatment duration (min)	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
0	100	51	100	15	74	0	90	0
60	99	70	100	80	48	8	55	30
120	99	88	100	90	58	37	70	30
180	99	91	100	90	59	7	100	70
240	93	84	100	95	61	13	90	80
300	100	82	100	80	53	12	65	40

Discussion

This work demonstrated that it is possible to cryopreserve apices sampled on bulbils and cloves of garlic using a vitrification protocol including treatment with the PVS3 vitrification solution. However, the regrowth rates achieved were lower with apices from bulbils than from cloves.

In our experiments, only treatment of apices with the PVS3 vitrification solution allowed regrowth of apices after cryopreservation, whereas no positive results were obtained after treatment with the PVS2 solution. Treatment with PVS2 has been successful with the majority of materials which have been cryopreserved using the vitrification technique (15, 16). The PVS3 vitrification solution has proven its efficiency with some materials including asparagus and strawberry cell suspensions (12, 21.) and apple apices (22) for which PVS2 was toxic. In their experiments with garlic apices, both Niwata (13) and Hannan and Garoutte (5) obtained positive results with the PVS2 vitrification solution but did not test other cryoprotective solutions. The differences noted as regards the toxicity of the cryoprotectants employed may be explained by the fact that different genotypes were employed in these studies. In addition, these authors worked with apices from cloves, which seem to be more resistant to cryopreservation than apices from bulbils, as also shown in the present study. The optimal duration of treatment with the cryoprotective solution varied with the genotype. Regrowth after freezing was highest after treatment durations between 120 and 240 min. Thus, it should be recommended, when cryopreserving new material to systematically experiment treatment durations of 120, 180 and 240 min.

A strong genotypic effect as well as an influence of the size of the bulbils on the regrowth potential were noted in our experiments, since cryopreservation was not successful with apices sampled from small bulbils. Further research is necessary to explain the reasons for these differences, which may be due to differences in the explant isolation procedure or the higher sensitivity of the meristems from small bulbils.

It has been shown in this study that apices from cloves have a higher regrowth rate than those from bulbils. However, cloves have three disadvantages as source of apices: 1) they are usually more infested with soil-borne microorganisms than bulbils; 2) explants are more difficult to isolate; and 3) the number of cloves and, thus, that of apices available for cryopreservation is usually much lower than that of bulbils for a given accession. However, cloves are the only source of explants for genotypes which produce no or a very limited set of bulbils.

In conclusion, additional experiments should be performed to optimize the cryopreservation protocol developed in this study, especially for apices sampled on small bulbils. Other vitrification solutions could be experimented with such as the PVS4 solution (16) which contains 35% glycerol, 20% ethylene glycol (w/v) and 0.6M sucrose, or the solution developed by Steponkus' group (9) which comprizes 40% ethylene glycol, 15% sorbitol and 6% bovine serum albumin. Other cryopreservation techniques such as encapsulation-dehydration or encapsulation-vitrification, which have been successfully applied to large number of species (3, 4) could also be tested.

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