CRYOPRESERVATION OF YOUNG INFLORESCENCE BASES IN BOLTING GARLIC FOR GERMPLASM STORAGE
(AEGIS Project)
21. 4. 2010 – 31. 12. 2010

Project Leader

Dr. E. R. Joachim Keller
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK)
Corrensstr. 3, 06466 Gatersleben
Germany

Project Partners

Dr. Teresa Kotlinska
Plant Genetic Resources Laboratory, Research Institute of Vegetable Crops (RIVC),
Konstytucji 3 Maja 1/3, 96-100, Skierniewice
Poland

Ms. Aida Reis
Banco Português de Germoplasma Vegetal, Unidade de Recursos Genéticos,
Ecofisiologia e Melhoramento de Plantas, INRB, I.P.,
Quinta de S. José, 4700-859, S. Pedro de Merelim, Braga
Portugal

BIOVERSITY INTERNATIONAL REPORT FACT SHEET

Library Report No. (Library use only)	

All required sections of this Fact Sheet are **completed by the Author of the** *Commissioned Organization or Contractor***.** Once completed, this Fact Sheet should be attached to the front of the report.

FULL TITLE of REPORT/PROJECT	Cryopreservation of young inflorescence bases in bolting garlic for germplasm storage	
AUTHOR of *Commissioned Organization or Contractor (Name and title of person)*	Dr. E. R. Joachim Keller	
NAME/ADDRESS of *Commissioned Organization or Contractor*	Ms S.-A. Lorenz and Prof. A. Graner, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstrasse 3, 06466 Gatersleben, Germany	
DATE REPORT SUBMITTED	31. 1. 2011	
TYPE OF REPORT *i.e. progress/final*	Final Report	
PREVIOUS REPORTS (Please fill in dates and add more lines if necessary) *To be completed by BIOVERSITY INTERNATIONAL Coordinator*	1st Progress Report	Date: no report (AEGIS coordinator informed) *(within 6th months after start of contract)*
	Final Report	Date: 31. 1. 2011 (*within one month after end of contract)*
BIOVERSITY INTERNATIONAL LETTER OF AGREEMENT *To be completed by BIOVERSITY INTERNATIONAL Coordinator*	10/049	
BIOVERSITY INTERNATIONAL PROJECT CODE *To be completed by BIOVERSITY INTERNATIONAL Coordinator*		
BIOVERSITY INTERNATIONAL CONTACT	J. Engels – AEGIS Coordinator	
ABSTRACT *(Minimum 100 words)*	Cryopreservation is the safest and most cost-effective method to maintain vegetatively propagated germplasm. Garlic falls into this category. So far cryopreservation is relying on bulbils, basal plates of *in vivo* and *in vitro* material. Additionally to this, a novel source can be used from the bases of unripe inflorescences. Within the AEGIS system, a small project was completed which aims at adopting this new method to genebank material under European conditions and at increasing effectiveness of cryopreservation in bolting garlic. Using unripe inflorescence bases, usability of mother plants is expanded, *in vitro* preculture can be omitted and the risk to lose mother plants during preparation is diminished. Three European genebanks (IPK Gatersleben, Germany; RIVC Skierniewice, Poland; BPGV Braga, Portugal) compared the various steps of the vitrification and droplet-vitrification protocols and optimised the procedure. Three clones fulfilling the requirements of a Most Appropriate Accession were selected as standard material and were investigated by all partners according to the method described by Kim et al. (2007). In the accession from the German collection, the	

	best regeneration after rewarming from cryopreservation, which was obtained in all three laboratories, amounted to rates between 75 % and 94 %. Comparing the two cryopreservation methods, droplet-vitrification was more effective than vitrification. Using inflorescences of different developmental stages higher regrowth rates were obtained for the older ones. Furthermore, three different durations (2 days, 4 weeks and 6 weeks) were tested in order to explore the best-suited time for cold storage of young inflorescences. Finally different durations of the pretreatments with PVS3 solution and the use of other PVS were tested.
KEYWORDS	Country/Region: Europe Crop(s): *Allium* Subject: droplet vitrification, PVS, *Allium sativum,* European project, cold pretreatment

AEGIS Project - Final report

Introduction:

Cryopreservation is the safest and most cost-effective method to maintain vegetatively propagated germplasm. Garlic (*Allium sativum*) falls into this category. Different source organs, like cloves, bulbils, basal plates of *in vivo* and *in vitro* material can be used for cryopreservation. Cloves have the disadvantage that they are mostly present in low quantities only; and they are, as organs taken from soil, often severely contaminated. When using *in vitro* plantlets a long preculture phase is necessary and the clones to be used as donor material cannot be safely maintained for more than 2 years. After longer time the quality of *in vitro* plants is more and more declining. For smaller bulbils of the *Longicuspis* type regeneration rates after cryopreservation were very low in most cases. In contrast to this, a novel source can be used from the bases of unripe inflorescences from *in vivo* material. Using unripe inflorescence bases, the time span is expanded in which explants can be taken from the mother plants and the time required for the protocol is reduced by skipping the *in vitro* multiplication phase.
The main objective of the project consists in the adoption of a new cryopreservation method by using unripe inflorescences as source organs according to the method described by Kim et al. (2007). Three European genebanks (IPK Gatersleben, Germany; RIVC Skierniewice, Poland; BPGV Braga, Portugal) selected five accessions fulfilling the requirements of a Most Appropriate Accession and exchanged three reference accessions to compared the various steps of the vitrification and droplet-vitrification protocols. Furthermore, three different developmental stages of inflorescences and three different durations of cold storage (2 days, 4 and 6 weeks) were tested in order to increase the effectiveness of cryopreservation in bolting garlic in the collaborating laboratories. Finally, other durations of the pretreatments with PVS3 solution and the use of other PVS were tested.

Material and Methods:

The basis material for cryopreservation was young inflorescences directly taken from the field. Selected bolting garlic accessions, received from the partners, were planted in the field. Donor material was taken from accessions which had been planted in the fields preliminarily in October / November 2009. At Gatersleben, two German accessions (All 0232; All 0514), each with 17 cloves, three Polish (171K; 148K; 350K), each with 30 cloves, and two Portuguese accessions (7123 and 7817) with 28 and 34 cloves, respectively, had been planted. Additional material was taken from the general garlic genebank field at IPK. At RIVC, five Polish bolting garlic accessions with 50 cloves of each, two German (see above) with 25 cloves of each and two Portuguese accessions (see above) with 26 and 30 cloves, respectively, had been planted. At BPGV, the five Portuguese, the two German and three Polish accessions had been previously planted in the field.
The total list of the accessions used in this research was given in Annex 1. The following three accessions selected from genebank collections of the three partners were defined as references. They were investigated by all three partners according to the standard cryopreservation method described by Kim et al (2007):

- All 0232 from Germany,
- 348 K from Poland,
- 7817 from Portugal.

The inflorescences of the appropriate, namely unripe, stage, which were grown to a distance of 5 - 10 cm above the uppermost leaf sheath, were cut and stored in a refrigerator for variable times. The harvest times are given in the respective tables of the experiments. For introduction, the inflorescences were sterilized by washing for 10 - 20 s in 70 % ethanol and subsequently placing in sodium hypochlorite solution (effective chlorine concentration 3 %), with 2 - 3 drops of Tween 20 for 12 min by shaking followed by 4 - 5 times rinsing with sterilized water. The spathes were removed by using a dissection microscope and explants were trimmed to a size of 1 mm in diameter to 2 mm in length including a piece of the inflorescence basis (according to Kim, it should include 2 - 3 bulbil primordia). Depending on the stage of the inflorescence 8 - 10 explants per inflorescence could be obtained. Minimum numbers were needed of 20 explants for the -LN control (full procedure excluding liquid nitrogen) and the +LN variants (full procedure) each and 5 - 10 explants for the growth control without treatment.

For pretreatment the explants were inoculated on medium 1: MS (Murashige and Skoog, 1962) + 0.3 mg/l indole acetic acid (IAA) + 2.0 mg/l 2-isopentenyladenine (2-iP) + 0.3 M sucrose + 9.5 g/l agar and cultured for 2 days at 10 °C (16 h light / 8 h dark). After this step, the first 5 - 10 explants were taken as growth control. The were transferred to medium 2: MS + 0.3 mg/l IAA + 2.0 mg/l 2-iP + 0.09 M sucrose + 9.5 g/l agar. The final explants were excised and pretreated with loading solution for 50 min followed by cryoprotectant mixture for different times (0.5 to 2.5 h) by constant shaking (80 rpm). Then, depending on the protocol, the explants were placed into cryoprotectant droplets adhering to an aluminium foil (droplet vitrification) or floated in the cryoprotectant solution in tubes (vitrification). They were rapidly cooled down to liquid nitrogen and stored there for 2 h. In the experiments the samples were quickly rewarmed in a water bath at 40 °C (vitrification) or by directly plunging in ambient-tempered unloading solution (droplet-vitrification). After a washing phase, they were cultivated at 24 °C in light on medium 2. Survival was counted 2 and 4 weeks, regeneration 10 weeks after rewarming.

During the start-up meeting hold on May 2, 2010 at IPK, the protocol was discussed in detail, which was followed by practical demonstration of the preparation of explants. Furthermore, a work scheme was elaborated and the experiments were planned accordingly for the three laboratories.

The following experiments were conducted:

I) *Standard experiment:* using the three agreed accessions by all partners.
   The parameters were organised according to Kim's publication:
   - Method: droplet-vitrification
   - Inflorescence stage according to picture in Annex 2B and letter K of the schemes (see Annex 2; B)
   - Cold storage for 4 weeks
   - PVS3 incubation for 2.5 h
   - PVS3 composition original as in the literature

II) *Different methods: d*roplet vitrification vs. vitrification (method see Makowska et al., 1999), however, the incubation solutions (incubation, loading, PVS3, unloading) as used by Kim et al. (2007).

III) *Different inflorescence stages*: three stages according to letters A (very young stage), K (middle stage) and O (old stage) were used (see pictures Annex 2)

IV) *Different storage durations*: without storage (only overnight-keeping); 4 weeks and 6 weeks storage at 5 °C

V) *Different incubation times of PVS3*: 0.5; 1; 2.5 h

VI) *Different PVS compositions*: comparison of the three standard PVS mixtures, which were published in the literature
- PVS2 (30 % glycerol + 15 % ethylene glycol + 15 % DMSO): 45 min on ice (0 °C) (Sakai et al., 1990)
- PVS3 (50 % sucrose + 50 % glycerol): 2.5 h at room temperature
- PVS4 (35 % glycerol + 20 % ethylene glycol + 0.6 M sucrose) 2.5 h at room temperature (Sakai, 2000).

All experiments were performed in two replications.
Additionally at RIVC, other three experiments were conducted to confirm the effectiveness of the droplet-vitrification method. In these additional experiments other three Polish accessions (171K, 298K, 509K) were used. For comparing the different methods additional accessions were also tested at BPGV.

Results:

At RIVC and BPGV, the best results of cryopreservation were obtained for the German accession All 0232 with regrowth of 94.0 and 78.0 %, respectively, but, with the χ^2 test, not significantly different to 74 %, which was obtained at IPK (see table 1). In contrast to that, at IPK the best regeneration results (87.9 %) were obtained for the Portuguese accession 7817, which showed the lowest regrowth rate in the other institutes. At IPK, however, the Polish accession had the lowest regrowth rate (51.4 %) in comparison to the other accessions. The other institutes attained only regenerations of 38.0 % and 48.0 %, respectively, for the Polish accession 348K.
This showed very clearly, that the differences of cryopreservation results between different accessions depend not only on the genotype, but also other components are important, e. g. the growing conditions, the plant vigour and personal peculiarities in preparation of the explants. However, in most cases the regeneration from unripe inflorescence explants was higher than found in former experiments using bulbils or *in vitro* plantlets.
Three additional experiments were carried out at RIVC using other three Polish garlic accessions (171K, 298 K, 509K) according to the standard method (table 2). Depending on the accessions the regeneration varied between 12 and 68 %. Interestingly in all three cases the regrowth rates were higher for +LN than for –LN. Similar observations were made also in other experiments done at RIVC and IPK, but not found in the experiments done at BPGV.

Table 1: Results of experiment I – Standard experiment

Accession number	Institute	Date of inflorescence harvest	Date of cryopreservation	Treatment	Survival (%)	Regrowth (%)
348K	IPK	07.06.2010	05.07.2010	-LN	55.41	63.51
				+LN	55.41	51.35
				growth control	100.00	100.00
	RIVC	02.06.2010	25.06.2010	-LN	5.00	5.00
				+LN	38.00	38.00
				growth control	100.00	100.00
	BPGV	10.05.2010	28.06.2010	-LN	82.50	67.50
				+LN	54.00	48.00
				growth control	100.00	100.00
ALL0232	IPK	04.06.2010	05.07.2010	-LN	66.13	70.97
				+LN	66.67	75.00
				growth control	100.00	100.00
	RIVC	26.05.2010	24.06.2010	-LN	72.50	75.00
				+LN	94.00	94.00
				growth control	100.00	100.00
	BPGV	10.05.2010	28.06.2010	-LN	80.00	72.50
				+LN	88.00	78.00
				growth control	100.00	100.00
7817*	IPK	22.06.2010	21.07.2010	-LN	83.33	91.67
				+LN	71.60	88.89
				growth control	100.00	100.00
	RIVC	18.06.2010	21.07.2010	-LN	25.00	25.00
				+LN	16.00	12.00
				growth control	100.00	100.00
	BPGV	04.06.2010	28.06.2010	-LN	47.50	52.50
				+LN	32.00	36.00
				growth control	70.00	60.00

* At RIVC only one experiment performed, because they had not enough plant material for a repetition

Table 2: Additional experiments done by RIVC – Standard method

Acc. no.	Date of inflorescence harvest	Date of cryopreservation	Treatment	Survival (%)	Regrowth (%)
298K	09.06.2010	22.07.2010	-LN	12.50	20.00
			+LN	24.00	68.00
			growth control	100.00	100.00
509K	14.06.2010	16.07.2010	-LN	35.00	15.00
			+LN	38.00	24.00
			growth control	100.00	100.00
171K	14.06.2010	22.07.2010	-LN	7.50	2.50
			+LN	18.00	12.00
			growth control	100.00	100.00

Comparing two cryopreservation methods, eight different accessions were tested by the three institutes. As visible in table 3 significantly (proven by the χ^2 test) higher regrowth rates were obtained by using the droplet-vitrification method, which were in average 56.9 % instead of 18.1% by using the vitrification method. Therefore, droplet-vitrification was more effective than vitrification. At IPK and BPGV, other accessions were used for testing of the different methods. However, the two accessions tested by RIVC were also the standard accessions All 0232 and 348K. The significant regrowth difference (proven by the χ^2 test) between these two accessions as found in experiment I was also measured by using the vitrification method. This confirms the better regeneration capacity of the German accession All 0232 in comparison to the Polish accession 348K detected also in the standard experiment I. Extreme differences were found for the Portuguese accessions, which regenerated with 68 % and 62 %, respectively, using the droplet-vitrification but only with 10 and 6 % using the vitrification method.

In the analysis of the different developmental stages of the inflorescences, no significant (χ^2 test-proven) differences between the stages K (middle age, Annex 2, Fig. 2) and O (old stage, Annex 2, Fig. 3) were observed in all three institutes (Table 4). At IPK, no significant differences to regeneration in stage A (very young stage, Annex 2, Fig. 1) were observed either. In contrast to that, significantly lower regrowth rates were obtained at RIVC and BPGV for stage A, and the worst results were got again for the Polish standard accession 348K. The best results of regeneration were found in all three institutes by using the old inflorescence stage O.

In the experiments about different storage durations, ambiguous results were found (table 5). At RIVC, only variants without storage or with 2 days of storage were effective with regrowth of 60 %. The remaining two other periods of storage duration, 4 and 6 weeks, respectively, were significantly lower in regeneration (proven by the χ^2 test). There, the regrowth rates were only 13 and 8 %, respectively. In contrary to that, at IPK the inflorescences stored for 6 weeks at 5 °C revealed the best regeneration of 95 %. This was significantly higher than for the variants of 4 weeks and 2 days storage (proven by the χ^2 test). On the other hand, no significant differences were found at BPGV. All in all, the regeneration of the German accession All 0766 used by IPK was much higher than of the two Polish accessions tested at the other two institutes.

The differences between the various incubation times within PVS3 solution ranging from 0.5 to 2.5 h were not significant (proven by the χ^2 test). Interestingly, the slightly higher regenerations were found for the short incubation time of 0.5 h and 1 h at IPK and RIVC. In opposite to this, at BPGV the higher regrowth rates were obtained for the 2.5 h incubation time of PVS3 used for the standard protocol.

The results obtained for the different compositions of PVS solutions were also not unambiguous (table 7). At IPK, significantly lower regeneration results (6.67 %) were detected for PVS2 in comparison to PVS3 and PVS4 which showed both nearly the same regrowth rates (28.33 and 25.42 %, respectively), whereas it revealed that PVS2 and PVS3 gave very similar results of regrowth, and this on a very low level (12 and 13 %, respectively) at RIVC. PVS4, however, was completely ineffective. Due to contamination within the Portuguese accession 7123, the second repetition was missing for the results of BPGV. Therefore, only results of one experiment are given in table 7. Nevertheless, the same results were detected at BPGV as found at RIVC, even, that PVS4 was totally ineffective. For PVS3, a little bit higher regeneration in comparison to PVS2 was obtained.

Table 3: Results of experiment II – Different methods

Method	Institute	Acc. no.	Date of inflorescence harvest	Date of cryo-preservation	Treatment	Survival (%)	Regrowth (%)
droplet-vitrification	IPK	171K	11.06.2010	22.07.2010	-LN	89.66	82.76
					+LN	83.33	71.21
					growth control	92.31	92.31
	RIVC	348K	02.06.2010	25.06.2010	-LN	5.00	5.00
					+LN	38.00	38.00
					growth control	100.00	100.00
		All 0232	26.05.2010	24.06.2010	-LN	72.50	75.00
					+LN	94.00	94.00
					growth control	100.00	100.00
	BPGV	All 0514	04.06.2010	21./22.06.2010	-LN	77.50	82.50
					+LN	74.00	54.00
					growth control	100.00	100.00
		7375	18.06.2010	21./22.06.2010	-LN	72.50	90.00
					+LN	58.00	68.00
					growth control	100.00	100.00
		7918	28.06.2010	19/20.07.2010	-LN	95.00	87.50
					+LN	84.00	68.00
					growth control	100.00	100.00
		6902	28.06.2010	19.07.2010	-LN	82.50	90.00
					+LN	42.00	62.00
					growth control l	100.00	100.00
vitrification	IPK	171K	11.06.2010	22.07.2010	-LN	60.00	40.00
					+LN	59.15	45.07
					growth control	100.00	100.00
	RIVC	348K	02.06.2010	30.06.2010	-LN	0.00	0.00
					+LN	10.00	10.00
					growth control	80.00	100.00
		All 0232	26.05.2010	24.06.2010	-LN	25.00	20.00
					+LN	54.00	64.00
					growth control	100.00	100.00
	BPGV	All 0514	04.06.2010	21./22.06.2010	-LN	70.00	75.00
					+LN	10.00	4.00
					growth control	100.00	100.00
		7375	18.06.2010	21./22.06.2010	-LN	85.00	95.00
					+LN	2.00	6.00
					growth control	100.00	100.00
		7918	28.06.2010	19/20.07.2010	-LN	100.00	87.50
					+LN	56.00	10.00
					growth control	100.00	100.00
		6902	28.06.2010	19.07.2010	-LN	37.50	87.50
					+LN	4.00	6.00
					growth control	100.00	100.00

Table 4: Results of experiment III – Different inflorescence stages

Stage	Institute	Acc. no.	Date of inflorescence harvest	Date of cryo-preservation	Treatment	Survival (%)	Regrowth (%)
Stage A	IPK	All 0791	07.06.2010	14./15.07.2010	-LN	74.58	83.05
					+LN	70.77	89.23
					growth control	92.86	92.86
	RIVC	348K	02.06.2010	01.07.2010	-LN	2.50	0.00
					+LN	2.00	2.00
					growth control	88.89	100.00
	BPGV	171K	10.05.2010	05/06.07.2010	-LN	40.00	55.00
					+LN	32.00	30.00
					growth control	100.00	100.00
Stage K	IPK	All 0791	09.06.2010	14./15.07.2010	-LN	71.93	84.21
					+LN	73.44	87.50
					growth control	92.31	92.31
	RIVC	348K	02.06.2010	07.07.2010	-LN	15.00	5.00
					+LN	14.00	20.00
					growth control	100.00	100.00
	BPGV	171K	17.05.2010	05/06.07.2010	-LN	67.50	85.00
					+LN	64.00	72.00
					growth control	100.00	100.00
Stage O	IPK	All 0791	05.07.2010	12./13.08.2010	-LN	95.00	91.67
					+LN	96.67	90.00
					growth control l	100.00	100.00
	RIVC	348K	02.06.2010	08.07.2010	-LN	5.00	5.00
					+LN	30.00	26.00
					growth control	100.00	100.00
	BPGV	171K	04.06.2010	05/06.07.2010	-LN	80.00	85.00
					+LN	58.00	80.00
					growth control	100.00	100.00

Table 5: Results of experiment IV – Different storage durations

storage duration	Institute	Acc. no.	Date of inflorescence harvest	Date of cryo-preservation	Treatment	Survival (%)	Regrowth (%)
without storage	IPK	All 0766	07.06.2010	18.06.2010	-LN	56.00	80.00
					+LN	44.44	63.49
					growth control	100.00	100.00
	RIVC	244K	14.06.2010	17.06.2010	-LN	12.50	22.50
					+LN	58.00	60.00
					growth control	100.00	100.00
	BPGV	350K	11.06.2010	14./15.06.2010	-LN	85.00	72.50
					+LN	30.00	18.00
					growth control	100.00	100.00
4 weeks storage	IPK	All 0766	14.06.2010	14./15.07.2010	-LN	76.67	88.89
					+LN	58.56	67.57
					growth control	95.45	100.00
	RIVC	244K	14.06.2010	16.07.2010	-LN	2.50	0.00
					+LN	12.00	12.00
					growth control	100.00	100.00
	BPGV	350K	11.06.2010	12/13.07.2010	-LN	87.50	62.50
					+LN	60.00	36.00
					growth control	100.00	100.00
6 weeks storage	IPK	All 0766	21.06.2010	04./05.08.2010	-LN	98.33	96.67
					+LN	96.67	95.00
					growth control	100.00	100.00
	RIVC	244K	14.06.2010	28.07.2010	-LN	35.00	17.50
					+LN	24.00	8.00
					growth control	100.00	100.00
	BPGV	350K	11.06.2010	26/27.07.2010	-LN	75.00	55.00
					+LN	58.00	28.00
					growth control	100.00	90.00

Table 6: Results of experiment V – Different incubation times of PVS3

Incubations time of PVS3	Institute	Acc. no.	Date of inflorescence harvest	Date of cryo-preservation	Treatment	Survival (%)	Regrowth (%)
1 h	IPK	All 0514	14.06.2010	26./27.07.2010	-LN	76.00	38.00
					+LN	50.82	44.26
					growth control l	100.00	95.00
0.5 h	RIVC	348K	15.06.2010	14.07.2010	-LN	5.00	5.00
					+LN	40.00	42.00
					growth control	100.00	100.00
	BPGV	348K	10.05.2010	12./13.07.2010	-LN	92.50	50.00
					+LN	42.00	24.00
					growth control	100.00	80.00
1.5 h	IPK	All 0514	14.06.2010	26./27.07.2010	-LN	69.09	34.55
					+LN	58.33	30.00
					growth control	100.00	95.00
1 h	RIVC	348K	15.06.2010	14.07.2010	-LN	5.00	5.00
					+LN	28.00	34.00
					growth control	100.00	100.00
1.5 h	BPGV	348K	10.05.2010	12./13.07.2010	-LN	80.00	57.50
					+LN	54.00	40.00
					growth control	100.00	80.00
2.5 h	IPK	All 0514	21.06.2010	26./27.07.2010	-LN	75.93	31.48
					+LN	46.67	26.67
					growth control	100.00	95.00
	RIVC	348K	15.06.2010	15.07.2010	-LN	7.50	7.50
					+LN	24.00	24.00
					growth control	100.00	100.00
	BPGV	348K	10.05.2010	12./13.07.2010	-LN	72.50	70.00
					+LN	44.00	48.00
					growth control	100.00	100.00

Table 7: Results of experiment VI – Different compositions of PVS

Com-position of PVS	Institute	Acc. no.	Date of inflorescence harvest	Date of cryo-preservation	Treatment	Survival (%)	Regrowth (%)
PVS2	IPK	All 0514	14.06.2010	26./27.07.2010	-LN	77.78	48.89
					+LN	46.67	6.67
					growth control	100.00	95.00
	RIVC	244K	14.06.2010	22.07.2010	-LN	10.00	7.50
					+LN	12.00	12.00
					growth control	100.00	100.00
	BPGV*	7123	11.06.2010	26./27.07.2010	-LN	60.00	55.00
					+LN	56.00	32.00
					growth control	80.00	80.00
PVS3	IPK	All 0514	14.06.2010	28./29.07.2010	-LN	42.86	26.19
					+LN	48.33	28.33
					growth control	100.00	95.00
	RIVC	244K	14.06.2010	16.07.2010	-LN	2.50	0.00
					+LN	12.00	12.00
					growth control	100.00	100.00
	BPGV*	7123	11.06.2010	26./27.07.2010	-LN	90.00	80.00
					+LN	76.00	48.00
					growth control	100.00	100.00
PVS4	IPK	All 0514	21.06.2010	28./29.07.2010	-LN	39.13	26.09
					+LN	35.59	25.42
					growth control	100.00	95.00
	RIVC	244K	14.06.2010	22.07.2010	-LN	0.00	0.00
					+LN	0.00	0.00
					growth control	100.00	100.00
	BPGV*	7123	11.06.2010	26./27.07.2010	-LN	75.00	60.00
					+LN	0.00	0.00
					growth control	100.00	n. d.

*only one experiment due to infection in the second repetition; n. d. = non detected

All detailed results of the three institutes are given in Annexes 3 - 5. Additionally, some pictures of the plantlets regenerated after rewarming are presented in Annex 6. In general it was observed that the regenerates came out of the primary explants as bunches of little plantlets enabling quick multiplication. This had been also realized in a preliminary experiment done at IPK. Another advantage was that the preparation of the explants went much quicker than the preparation of ripe bulbils or *in vitro* plants needed. The new method is suitable for all germplasm of bolting garlic. The results obtained during realization of the AEGIS project are well usable but not always consistent in all investigated accessions. Nevertheless, the results obtained were, in many cases, as good as or sometimes even much better than those from bulbils or *in vitro* plants. Using the latter option requires a long multiplication phase. As this phase can be skipped, the entire procedure will be much shorter then when *in vitro* material is used. Thus, the overall benefits mainly consist in quicker introduction of material into cryopreservation also in the following option combining the use of inflorescences with that of ripe bulbils. The inflorescences are available from Mai to June and from October to March ripe bulbils can be used for cryopreservation.

Recommendations:

The adoption of a new cryopreservation method using unripe inflorescences of garlic as a new source of organs can be introduced in genebanks for cryopreservation of garlic germplasm.

When enough plant material of the respective bolting accession is available from the field, it could be possible to use ripe bulbils and unripe inflorescences successively together for cryopreservation. The innovation consists in the introduction of a new explant type into routine cryopreservation, which allows speeding up the procedures of cryopreservation by a new protocol.

Bibliography:

Kim, H.-H., Lee, J.-K., Hwang, H.-S. and Engelmann, F. 2007. Cryopreservation of garlic germplasm collections using the droplet-vitrification technique. CryoLetters 28: 471-482.

Makowska Z., Keller J. and Engelmann, F. 1999. Cryopreservation of apices isolated from garlic (*Allium sativum* L.) bulbils and cloves. CryoLetters 20: 175-182.

Maaß, H.I. and M. Klaas, M. 1995. Infraspecific differentiation of garlic (*Allium sativum* L.) by isozyme and RAPD markers. Theor. Appl. Genet. 91: 89-97.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum 15: 473-497.

Sakai, A., 2000. Development of cryopreservation techniques. In: Engelmann F. and Takagi, H. (eds) Cryopreservation of tropical plant germplasm – current research progress and applications. Proc JIRCAS/IPCRI Joint Int. Workshop, 20-23 Oct 1998, Tsukuba, Japan, pp 1-7.

Sakai, A., Kobayashi, S. and Oiyama, I. 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Rep. 9: 30-33.

Attachments:

Annex 1: Bolting garlic accessions of all partners used in project

Accession number	Used at	For the following Experiment	Subtaxa*	Acquisition year	Country of origin
All 0232	all institutes	Standard Accession	*Ophioscorodon*	1957	DEU
225 686 (348K)	all institutes	Standard Accession	*Longicuspis*	1997	POL
7817	all institutes	Standard Accession			PRT
All 0514	IPK	Different Incubation time in PVS 3; Different Composition of PVS	*Ophioscorodon*	1975	DEU
All 0766	IPK	Different Storage Duration	*Longicuspis*	1983	GEO
All 0791	IPK	Different Inflorescence Stages	*Longicuspis*	1986	GEO
225 590 (171K)	IPK	Different Methods	*Longicuspis*	1990	RUS
225 551 (244K)	RIVC	Different Storage Duration; Different Composition of PVS	*Longicuspis*	1991	POL
225 686 (348K)	RIVC	Different Methods; Different Inflorescence Stages; Different Incubation time in PVS 3	*Longicuspis*	1997	POL
ALL 0232	RIVC	Different Methods	*Ophioscorodon*	1957	DEU
225 652 (298K)	RIVC	Additional experiments with standard method	*Longicuspis*	1988	UZB
225 590 (171K)	RIVC	Additional experiments with standard method	*Longicuspis*	1990	RUS
7375	BPGV	Different Methods		1998	PRT
7918	BPGV	Different Methods		2000	PRT
6902	BPGV	Different Methods		1996	PRT
7123	BPGV	Different Composition of PVS		1997	PRT
All 514	BPGV	Different Methods	*Ophioscorodon*	1975	DEU
348 K	BPGV	Different Incubation time in PVS 3	*Longicuspis*	1997	POL
350 K	BPGV	Different Storage Duration			POL
171K	BPGV	Different Inflorescence Stages	*Longicuspis*	1990	RUS

Annex 2: Definition of the different inflorescence stages

A) Stage A, *Longicuspis* type
All 0499, year 1998, May 25, week 22

Stage A, *Ophioscorodon* type
All 1165, year 1998, May 18, week 21

B) Stage K, *Ophioscorodon* type
All 0499, year 1998, June 8, week 24

Stage K, *Longicuspis* type
All 1165, year 1998, May 25, week 22

C) Stage O, *Ophioscorodon* type
All 0499, year 1998, June 12, week 25

Stage O, *Longicuspis* type
All 1165 year 1998, June 12, week 25

Annex 3: Detailed results of IPK

A) Experiment I – Standard experiment

			1. Evaluation 2 weeks after rewarming						**2. Evaluation** 4 weeks after rewarming					**3. Evaluation** 10 weeks after rewarming				
Acc. no.	Date of cryo-preservation	treat-ment	No. of explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate 1 (%)	Date of obser-vation	No. of green explants	No. of died explants	No.of infected explants	Survival rate 2 (%)	Date of obser-vation	No. of plantlets	No. of died explants	No.of infected explants	Re-growth rate (%)	Date of obser-vation
All 0232 / I	07.07.10	- LN	32	19	13	0	59.38	21.07.10	18	14	(6)	56.25	04.08.10	20	12	0	62.50	15.09.10
		+ LN	30	20	10	0	66.67		24	6	0	80.00		21	9	0	70.00	
		growth control	7	7	0	0	100.00		7	0	0	100.00		7	0	0	100.00	
All 0232 / II	08.07.10	- LN	30	22	8	0	73.33	22.07.10	25	5	(7)	83.33	04.08.10	24	6	0	80.00	16.09.10
		+ LN	30	20	10	0	66.67		25	5	0	83.33		24	6	0	80.00	
		growth control	9	9	0	0	100.00		9	0	0	100.00		9	0	0	100.00	
348K / I	07.07.10	- LN	40	20	20	0	50.00	21.07.10	23	17	0	57.50	05.08.10	22	18	0	55.00	15.09.10
		+ LN	40	20	20	0	50.00		17	23	0	42.50		19	21	0	47.50	
		growth control	6	6	0	0	100.00		6	0	0	100.00		6	0	0	100.00	
348K / II	08.07.10	- LN	34	21	13	0	61.76	22.07.10	29	5	0	85.29	05.08.10	25	9	3	73.53	16.09.10
		+ LN	34	21	13	0	61.76		22	12	(3)	64.71		19	15	3	55.88	
		growth control	8	8	0	0	100.00		8	0	0	100.00		8	0	8	100.00	
7817 / I	23.07.10	- LN	30	23	7	3	76.67	06.08.10	25	5	(18)	83.33	20.08.10	25	5	0	83.33	01.10.10
		+ LN	40	31	9	6	77.50		31	9	(12)	77.50		32	8	(4)	80.00	
		growth control	7	7	0	0	100.00		7	0	0	100.00		7	0	0	100.00	
7817 / II	23.07.10	- LN	30	27	3	9	90.00	06.08.10	30	0	(19)	100.00	20.08.10	30	0	0	100.00	01.10.10
		+ LN	41	27	14	6	65.85		40	1	(13)	97.56		40	1	0	97.56	
		growth control	10	10	0	2	100.00		10	0	(8)	100.00		10	0	0	100.00	

Annex 3: Detailed results of IPK, continued

B) Experiment II – Different methods

			1. Evaluation 2 weeks after rewarming					**2. Evaluation** 4 weeks after rewarming				**3. Evaluation** 10 weeks after rewarming				
Acc. no.	Date of cryopre-servation	treatment	No. of explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate 1 (%) on 04./05.08.10	No. of green explants	No. of died explants	No.of infected explants	Survival rate 2 (%) on 18./19.08.10	No. of plantlets	No. of died explants	No.of infected explants	Regrowth rate (%) on 29./30.08.10	
171K / I	21.07.10	- LN	30	28	2	0	93.33	26	4	0	86.67	26	4	0	86.67	**droplet-vitrifi-cation**
		+ LN	30	25	5	0	83.33	25	6	0	83.33	26	4	0	86.67	
		growth control	5	4	1	1	80.00	4	1	0	80.00	4	1	0	80.00	
171K / II	22.07.10	- LN	28	24	4	0	85.71	24	4	0	85.71	22	6	0	78.57	
		+ LN	36	30	6	1	83.33	21	15	1	58.33	21	15	0	58.33	
		growth control	8	8	0	2	100.00	8	0	0	100.00	8	0	0	100.00	
171K / I	21.07.10	- LN	30	19	11	0	63.33	13	17	0	43.33	15	15	0	50.00	**vitrifi-cation**
		+ LN	30	17	13	0	56.67	12	18	0	40.00	12	18	4	40.00	
		growth control	5	5	0	1	100.00	5	0	0	100.00	5	0	0	100.00	
171K / II	22.07.10	- LN	30	17	13	14	56.67	9	21	19	30.00	9	21	19	30.00	
		+ LN	41	25	16	0	60.98	20	21	0	48.78	20	21	0	48.78	
		growth control	8	8	0	3	100.00	8	0	0	100.00	8	0	0	100.00	

Annex 3: Detailed results of IPK, continued

C) Experiment III – Different inflorescence stages

			1. Evaluation 2 weeks after rewarming						**2. Evaluation** 4 weeks after rewarming					**3. Evaluation** 10 weeks after rewarming					
Acc. no.	Date of cryo-preser-vation	treat-ment	No. of explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate 1 (%)	Date of obser-vation	No. of green explants	No. of died explants	No.of infected explants	Survival rate 2 (%)	Date of obser-vation	No. of plantlets	No. of died explants	No.of infected explants	Regrowth rate (%)	Date of obser-vation	
All 0791 / I	14.07.10	- LN	29	22	7	0	75.86	28.07.10	23	6	0	79,31	11.08.10	24	5	3	82.76	22.09.10	**stage A**
		+ LN	29	21	8	0	72.41		23	6	0	79,31		26	3	0	89.66		
		growth control	6	6	0	0	100.00		6	0	0	100,00		6	0	3	100.00		
All 0791 / II	15.07.10	- LN	30	22	8	0	73.33	29.07.10	25	5	0	83,33	12.08.10	25	5	0	83.33	23.09.10	
		+ LN	36	25	11	0	69.44		32	4	0	88,89		32	2	0	88.89		
		growth control	8	7	1	0	87.50		7	1	0	87,50		7	1	0	87.50		
All 0791 / I	14.07.10	- LN	30	23	7	0	76.67	28.07.10	25	5	0	83,33	11.08.10	27	3	0	90.00	22.09.10	**stage K**
		+ LN	34	28	6	0	82.35		32	2	0	94,12		32	2	0	94.12		
		growth control	7	7	0	0	100.00		7	0	0	100,00		7	0	0	100.00		
All 0791 / II	15.07.10	- LN	27	18	9	0	66.67	29.07.10	18	9	0	66,67	12.08.10	21	6	3	77.78	23.09.10	
		+ LN	30	19	11	0	63.33		18	12	0	60,00		24	6	0	80.00		
		growth control	6	5	1	0	83.33		5	1	0	83,33		5	1	0	83.33		
All 0791 / I	12.08.10	- LN	30	28	2	0	93.33	26.08.10	27	3	0	90,00	09.09.10	27	3	0	90.00	21.10.10	**stage O**
		+ LN	30	30	0	0	100.00		25	5	0	83,33		24	6	0	80.00		
		growth control	5	5	0	0	100.00		5	0	0	100,00		5	0	0	100.00		
All 0791 / II	13.08.10	- LN	30	29	1	0	96.67	27.08.10	29	1	0	96,67	10.09.10	28	2	0	93.33	22.10.10	
		+ LN	30	28	2	0	93.33		28	2	0	93,33		30	0	0	100.00		
		growth control	5	5	0	0	100.00		5	0	0	100,00		5	0	0	100.00		

Annex 3: Detailed results of IPK, continued

D) Experiment IV – Different storage durations

			1. Evaluation 2 weeks after rewarming						**2. Evaluation** 4 weeks after rewarming					**3. Evaluation** 10 weeks after rewarming					
Acc. no.	Date of cryo-preser-vation	treat-ment	No. of explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate 1 (%)	Date of obser-vation	No. of green explants	No. of died explants	No.of infected explants	Survival rate 2 (%)	Date of obser-vation	No. of plantlets	No. of died explants	No.of infected explants	Regrowth rate (%)	Date of obser-vation	
All 0766 / I	18.06.10	- LN	23	12	11	0	52.17	30.06. / 02.07.10	15	8	0	65.22	21.07.10	19	4	0	82.61	27.08.10	**0 - 2 days**
		+ LN	32	15	17	0	46.88		18	14	0	56.25		24	8	0	75.00		
		growth control	5	5	0	0	100.00		5	0	0	100.00		5	0	0	100.00		
All 0766 / II	18.06.10	- LN	27	16	11	0	59.26	30.06. / 02.07.10	19	8	0	70.37	21.07.10	21	6	0	77.78	27.08.10	
		+ LN	31	13	18	0	41.94		15	16	0	48.39		16	15	0	51.61		
		growth control	5	5	0	0	100.00		5	0	0	100.00		5	0	0	100.00		
All 0766 / I	14.07.10	- LN	40	27	13	0	67.50	28.07.10	33	7	0	82.50	11.08.10	34	6	0	85.00	22.09.10	**4 weeks**
		+ LN	50	27	23	0	54.00		29	21	0	58.00		29	21	1	58.00		
		growth control	10	9	1	0	90.00		10	0	0	100.00		10	0	0	100.00		
All 0766 / II	15.07.10	- LN	50	42	8	0	84.00	29.07.10	44	6	0	88.00	12.08.10	46	4	3	92.00	23.09.10	
		+ LN	61	38	22	0	62.30		45	16	0	73.77		46	15	6	75.41		
		growth control	12	12	0	0	100.00		12	0	0	100.00		12	0	0	100.00		
All 0766 / I	04.08.10	- LN	30	29	1	0	96.67	18.08.10	28	2	0	93.33	01.09.10	28	2	0	93.33	13.10.10	**6 weeks**
		+ LN	35	34	1	0	97.14		34	1	0	97.14		33	2	0	94.29		
		growth control	10	10	0	0	100.00		10	10	0	100.00		10	0	0	100.00		
All 0766 / II	05.08.10	- LN	30	30	0	0	100.00	19.08.10	30	0	0	100.00	02.09.10	30	1	0	100.00	14.10.10	
		+ LN	25	24	1	0	96.00		22	3	0	88.00		24	1	0	96.00		
		growth control	10	10	0	0	100.00		10	10	0	100.00		10	0	0	100.00		

Annex 3: Detailed results of IPK, continued

E) Experiment V – Different incubation times of PVS 3

			1. Evaluation 2 weeks after rewarming					**2. Evaluation** 4 weeks after rewarming				**3. Evaluation** 10 weeks after rewarming				
Acc. No.	Date of cryo-preservation	treat-ment	No. of explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate 1 (%) on 11./12.08.10	No. of green explants	No. of died explants	No.of infected explants	Survival rate 2 (%) on 25./26.08.10	No. of green explants	No. of died explants	No.of infected explants	Regrowth rate (%) on 06./07.10.10	
All 0514 / I	28.07.10	- LN	26	19	7	5	73.08	18	8	6	69.23	10	16	(6)	38.46	**1 h**
		+ LN	30	10	20	3	33.33	13	17	3	43.33	11	19	4	36.67	
All 0514 / II	29.07.10	- LN	24	19	5	0	79.17	14	10	0	58.33	9	15	3	37.50	
		+ LN	31	21	10	0	67.74	14	17	0	45.16	16	15	0	51.61	
All 0514 / I	28.07.10	- LN	30	20	10	0	66.67	12	18	0	40.00	8	22	0	26.67	**1 h 30 min**
		+ LN	30	14	16	2	46.67	9	21	3	30.00	6	24	2	20.00	
All 0514 / II	29.07.10	- LN	25	18	7	0	72.00	16	9	0	64.00	11	14	3	44.00	
		+ LN	30	21	9	0	70.00	11	19	2	36.67	10	20	(2)	33.33	
All 0514 / I	28.07.10	- LN	30	23	7	1	76.67	20	10	5	66.67	7	23	4	23.33	**2 h 30 min**
		+ LN	30	11	19	0	36.67	9	21	1	30.00	3	27	(1)	10.00	
All 0514 / II	29.07.10	- LN	24	18	6	0	75.00	14	10	0	58.33	10	14	1	41.67	
		+ LN	30	17	13	0	56.67	14	16	0	46.67	13	17	0	43.33	
All 0514 / I	28.07.10	growth control	10	10	0	10	100.00	10	0	10	100.00	9	1	1	90.00	
All 0514 / II	29.07.10		10	10	0	8	100.00	10	0	8	100.00	10	0	1	100.00	

Annex 3: Detailed results of IPK, continued

F) Experiment VI – Different PVS compositions

			1. Evaluation 2 weeks after rewarming					**2. Evaluation** 4 weeks after rewarming				**3. Evaluation** 10 weeks after rewarming				
Acc. No.	experiment date	treat-ment	No. of explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate 1 (%) on 11./12.08.10	No. of green explants	No. of died explants	No.of infected explants	Survival rate 2 (%) on 25.08.2010	No. of green explants	No. of died explants	No.of infected explants	Regrowth rate (%) on 06./07.10.10	
All 0514 / I	28.07.10	- LN	20	16	4	3	80.00	14	6	3	70.00	11	9	0	55.00	
		+ LN	30	12	18	0	40.00	6	24	0	20.00	0	30	3	0.00	**PVS2**
All 0514 / II	29.07.10	- LN	25	19	6	3	76.00	19	6	4	76.00	11	14	3	44.00	
		+ LN	30	16	14	0	53.33	3	27	0	10.00	4	26	0	13.33	
All 0514 / I	28.07.10	- LN	20	10	10	0	50.00	8	12	0	40.00	5	15	9	25.00	
		+ LN	30	19	11	0	63.33	14	16	10	46.67	13	17	0	43.33	**PVS3**
All 0514 / II	29.07.10	- LN	22	8	14	0	36.36	6	16	0	27.27	6	16	0	27.27	
		+ LN	30	10	20	0	33.33	7	23	1	23.33	4	26	0	13.33	
All 0514 / I	28.07.10	- LN	20	11	9	0	55.00	10	10	0	50.00	6	16	0	30.00	
		+ LN	30	11	19	0	36.67	12	18	0	40.00	9	21	0	30.00	**PVS4**
All 0514 / II	29.07.10	- LN	26	7	19	0	26.92	7	19	0	26.92	6	20	0	23.08	
		+ LN	29	10	19	0	34.48	9	20	4	31.03	6	23	0	20.69	
All 0514 / I	28.07.10	growth control	10	10	0	0	100.00	10	0	2	100.00	10	0	2	100.00	
All 0514 / II	29.07.10		10	10	0	4	100.00	10	0	4	100.00	9	1	1	90.00	

Annex 4: Detailed results of RIVC

A) Experiment I – Standard experiment

				Observation after 2 weeks				Observation after 2 months			
Standard Accessions	Date of cryo-preservation	treatment	No. Explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate (%)	No. of green explants	No. of died explants	No.of infected explants	Regeneration rate (%)
348K / I	25.06.10	- LN	20	1	19	0	5.0	1	19	0	5.0
		+ LN	25	7	18	0	28.0	11	14	0	44.0
		growth control	5	5	0	0	100.0	5	0	0	100.0
348K / II	25.06.10	- LN	20	1	19	0	5.0	1	19	0	5.0
		+ LN	25	12	13	0	48.0	8	17	0	32.0
		growth control	5	5	0	0	100.0	5	0	0	100.0
ALL 0232/I	24.06.10	- LN	20	15	5	0	75.0	16	4	0	80.0
		+ LN	25	23	2	0	92.0	23	2	0	92.0
		growth control	5	5	0	0	100.0	5	0	0	100.0
ALL 0232/II	24.06.10	- LN	20	14	6	0	70.0	14	6	0	70.0
		+ LN	25	24	1	0	96.0	24	1	0	96.0
		growth control	5	5	0	0	100.0	5	0	0	100.0
7817/I*	21.07.10	- LN	20	5	15	0	25.0	5	15	0	25.0
		+ LN	25	4	21	0	16.0	3	22	0	12.0
		growth control	5	5	0	0	100.0	5	0	0	100.0

Annex 4: Detailed results of RIVC, continued

B) Experiment II – Different methods

				Observation after 2 weeks				Observation after 2 months			
Vitrification method	Date of cryo-preservation	treatment	No. Explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate (%)	No. of green explants	No. of died explants	No.of infected explants	Regeneration rate (%)
348K / I	30.06.10	- LN	20	0	20	0	0	0	20	0	0.0
		+ LN	25	2	23	0	8.0	2	23	0	8.0
		growth control	5	5	0	0	100.0	5	0	0	100.0
348K / II	30.06.10	- LN	20	0	20	0	0.0	0	20	0	0.0
		+ LN	25	3	22	0	12.0	3	22	0	12.0
		growth control	5	3	2	0	60.0	5	0	0	100.0
ALL 0232 / I	24.06.10	- LN	20	5	15	0	25.0	3	17	0	15.0
		+ LN	25	13	12	0	52.0	17	8	0	68.0
		growth control	5	5	0	0	100.0	5	0	0	100.0
ALL 0232 / II	24.06.10	- LN	20	5	15	0	25.0	5	15	0	25.0
		+ LN	25	14	11	0	56.0	15	10	0	60.0
		growth control	5	5	0	0	100.0	5	0	0	100.0

Annex 4: Detailed results of RIVC, continued

C) Experiment III – Different inflorescence stages

				Observation after 2 weeks				Observation after 2 months				
Different inflorescence stages	Date of cryo-preservation	treatment	No. Explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate (%)	No. of green explants	No. of died explants	No.of infected explants	Regeneration rate (%)	
348K / I	01.07.10	- LN	20	1	19	0	5.0	0	20	0	0.0	**stage A**
		+ LN	25	1	24	0	4.0	1	24	0	4.0	
		growth control	4	4	0	0	100.0	4	0	0	100.0	
348K / II	01.07.10	- LN	20	0	20	0	0.0	0	20	0	0.0	
		+ LN	25	0	25	0	0.0	0	25	0	0.0	
		growth control	5	4	1	0	100.0	5	0	0	100.0	
348K / I	07.07.10	- LN	20	3	17	0	15.0	1	19	0	5.0	**stage K**
		+ LN	25	2	23	0	8.0	3	22	0	12.0	
		growth control I	5	5	0	0	100.0	5	0	0	100.0	
348K / II	07.07.10	- LN	20	3	17	0	15.0	1	19	0	5.0	
		+ LN	25	5	20	0	20.0	7	18	0	28.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
348K / I	08.07.10	- LN	20	1	19	0	5.0	1	19	0	5.0	**stage O**
		+ LN	25	8	17	0	32.0	6	19	0	24.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
348K / II	08.07.10	- LN	20	1	19	0	5.0	1	19	0	5.0	
		+ LN	25	7	18	0	28.0	7	18	0	28.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	

Annex 4: Detailed results of RIVC, continued

D) Experiment IV – Different storage durations

				Observation after 2 weeks				Observation after 2 months				
Different storage duration	Date of cryo-preservation		No. Explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate (%)	No. of green explants	No. of died explants	No.of infected explants	Regeneration rate (%)	
244K / I	17.06.10	- LN	20	3	17	0	15.0	4	16	0	20.0	**Only 2 days**
		+ LN	25	14	11	0	56.0	15	10	0	60.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
244K / II	17.06.10	- LN	20	2	18	0	10.0	5	15	0	25.0	
		+ LN	25	15	10	0	60.0	15	10	0	60.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
244K /I	16.07.10	- LN	20	1	19	0	5.0	0	20	0	0.0	**4 weeks**
		+ LN	25	2	23	0	8.0	2	23	0	10.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
244K /II	16.07.10	- LN	20	0	20	0	0.0	0	20	0	0.0	
		+ LN	25	4	21	0	16.0	4	21	0	16.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
244K /I	28.07.10	- LN	20	7	15	0	35.0	5	15	0	25.0	**6 weeks**
		+ LN	25	7	21	0	28.0	3	22	0	12.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
244K /II	28.07.10	- LN	20	7	13	0	35.0	2	18	0	10.0	
		+ LN	25	5	20	0	20.0	1	24	0	4.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	

Annex 4: Detailed results of RIVC, continued

E) Experiment V – Different incubation times of PVS 3

				Observation after 2 weeks				Observation after 2 months				
Different incubation of PVS3	Date of cryo-preservation		No. Explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate (%)	No. of green explants	No. of died explants	No.of infected explants	Regeneration rate (%)	
348K / II	14.07.10	- LN	20	1	19	0	5.0	1	19	0	5.0	**0.5 hour**
		+ LN	25	8	17	0	32.0	10	15	0	40.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
348K / II	14.07.10	- LN	20	1	19	0	5.0	1	19	0	5.0	
		+ LN	25	12	13	0	48.0	11	14	0	44.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
348K / II	14.07.10	- LN	20	1	19	0	5.0	1	19	0	5.0	**1 hour**
		+ LN	25	7	18	0	28.0	7	18	0	28.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
348K / II	14.07.10	- LN	20	1	19	0	5.0	1	19	0	5.0	
		+ LN	25	7	18	0	28.0	10	15	0	40.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
348K / II	15.07.10	- LN	20	2	18	0	10.0	2	18	0	10.0	**2.5 hours**
		+ LN	25	8	17	0	32.0	8	17	0	32.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
348K / II	15.07.10	- LN	20	1	19	0	5.0	1	19	0	5.0	
		+ LN	25	4	21	0	16.0	4	21	0	16.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	

Annex 4: Detailed results of RIVC, continued

F) Experiment VI – Different PVS compositions

				Observation after 2 weeks				Observation after 2 months				
Different composition of PVS	Date of cryo-preservation		No. Explants	No. of green explants	No. of died explants	No.of infected explants	Survival rate (%)	No. of green explants	No. of died explants	No.of infected explants	Regeneration rate (%)	
244K / I	22.07.10	- LN	20	2	18	0	10.0	1	19	0	5.0	**PVS2**
		+ LN	25	3	22	0	12.0	4	21	0	16.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
244K / II	22.07.10	- LN	20	2	18	0	5.0	2	18	0	10.0	
		+ LN	25	3	22	0	12.0	2	23	0	8.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
244K /I	16.07.10	- LN	20	1	19	0	5.0	0	20	0	0.0	**PVS3**
		+ LN	25	2	23	0	8.0	2	23	0	10.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
244K /II	16.07.10	- LN	20	0	20	0	0.0	0	20	0	0.0	
		+ LN	25	4	21	0	16.0	4	21	0	16.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
244K /I	22.07.10	- LN	20	0	20	0	0.0	0	20	0	0.0	**PVS4**
		+ LN	25	0	25	0	0.0	0	25	0	0.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	
244K /II	22.07.10	- LN	20	0	20	0	0.0	0	20	0	0.0	
		+ LN	25	0	25	0	0.0	0	25	0	0.0	
		growth control	5	5	0	0	100.0	5	0	0	100.0	

Annex 5: Detailed results of BPGV

A) Experiment I – Standard experiment

				1. Evaluation 2 weeks after rewarming			**2. Evaluation** 4 weeks after rewarming		**3. Evaluation** 10 weeks after rewarming	
Acc. No.	Collecting date	experiment date	treatment	No. of explants	No. of green explants	Survival rate 1 (%) on 16.07.2010	No. of green explants	Survival rate 2 (%) on 30.07.2010	No. of plantlets	Regrowth rate (%) on 30.08.2010
348K / I	10.05.10	28. / 29.06.2010	- LN	20	16	80.0	18	90.0	14	70.0
			+ LN	25	15	60.0	16	64.0	14	56.0
			growth control I	5	5	100.0	5	100.0	5	100.0
348K / II			- LN	20	17	85.0	17	85.0	13	65.0
			+ LN	25	12	48.0	13	52.0	10	40.0
			growth control	5	5	100.0	5	100.0	5	100.0
ALL 0232 / I			- LN	20	18	90.0	19	95.0	16	80.0
			+ LN	25	24	96.0	25	100.0	20	80.0
			growth control	5	5	100.0	5	100.0	5	100.0
ALL 0232 / II			- LN	20	14	70.0	16	80.0	13	65.0
			+ LN	25	20	80.0	21	84.0	19	76.0
			growth control	5	5	100.0	5	100.0	5	100.0
7817 / I	04.06.10		- LN	20	11	55.0	11	55.0	9	45.0
			+ LN	25	10	40.0	13	52.0	8	32.0
			growth control	5	3	60.0	3	60.0	3	60.0
7817 / II			- LN	20	8	40.0	12	60.0	12	60.0
			+ LN	25	6	24.0	11	44.0	10	40.0
			growth control	5	4	80.0	4	80.0	3	60.0

Annex 5: Detailed results of BPGV, continued

B) Experiment II – Different methods

				1. Evaluation 2 weeks after rewarming			**2. Evaluation** 4 weeks after rewarming		**3. Evaluation** 10 weeks after rewarming		
Acc. No.	Collecting date	experiment date	treatment	No. of explants	No. of green explants	Survival rate 1 (%) on 09.07.2010	No. of green explants	Survival rate 2 (%) on 23.07.2010	No of plantlets	Regrowth rate (%) on 30.08.2010	
All 514 / II	04.06.10	21./22.06.10	- LN	20	16	80.0	18	90.0	18	90.0	**droplet-vitrification**
			+ LN	25	17	68.0	20	80.0	15	60.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
All 514 / I			- LN	20	15	75.0	18	90.0	15	75.0	
			+ LN	25	20	80.0	16	64.0	12	48.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
All 514 / II			- LN	20	12	60.0	16	80.0	16	80.0	**Vitrification**
			+ LN	25	2	8.0	3	12.0	2	8.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
All 514 / I			- LN	20	16	80.0	17	85.0	14	70.0	
			+ LN	25	3	12.0	4	16.0	0	0.0	
			growth control	5	5	100.0	5	100.0	5	100.0	

Due to bacterial infection inside this accessions results not valuated

Annex 5: Detailed results of BPGV, continued

B) Experiment II – Different methods, continued

				1. Evaluation 2 weeks after rewarming			**2. Evaluation** 4 weeks after rewarming		**3. Evaluation** 10 weeks after rewarming		
Acc. No.	Collecting date	experiment date	treatment	No. of explants	No. of green explants	Survival rate 1 (%) on 09.07.2010	No. of green explants	Survival rate 2 (%) on 23.07.2010	No of plantlets	Regrowth rate (%) on 30.08.2010	
7375 / I	18.06.10	21/22.06.2010	- LN	20	15	75.0	18	90.0	17	85.0	**droplet-vitrifi-cation**
			+ LN	25	16	64.0	21	84.0	16	64.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
7376 / II			- LN	20	14	70.0	19	95.0	19	95.0	
			+ LN	25	13	52.0	18	72.0	18	72.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
7375 / I			- LN	20	19	95.0	20	100.0	20	100.0	**vitrifi-cation**
			+ LN	25	0	0.0	5	20.0	1	4.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
7376 / II			- LN	20	15	75.0	18	90.0	18	90.0	
			+ LN	25	1	4.0	3	12.0	3	12.0	
			growth control	5	5	100.0	5	100.0	5	100.0	

Annex 5: Detailed results of BPGV, continued

B) Experiment II – Different methods, continued

				1. Evaluation 2 weeks after rewarming			**2. Evaluation** 4 weeks after rewarming		**3. Evaluation** 10 weeks after rewarming		
Acc. No.	Collecting date	experiment date	treatment	No. of explants	No. of green explants	Survival rate 1 (%) on 30.07.2010	No. of green explants	Survival rate 2 (%) on 16.08.2010	No of plantlets	Regrowth rate (%) on 27.09.2010	
7918 / I	28.06.10	19/20.07.2010	- LN	20	19	95.0	19	95.0	18	90.0	**droplet-vitrification**
			+ LN	25	22	88.0	22	88.0	16	64.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
7918 / II			- LN	20	19	95.0	18	90.0	17	85.0	
			+ LN	25	20	80.0	19	76.0	18	72.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
7918 / I			- LN	20	20	100.0	20	100.0	18	90.0	**vitrification**
			+ LN	25	12	48.0	7	28.0	3	12.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
7918 / II			- LN	20	20	100.0	18	90.0	17	85.0	
			+ LN	25	16	64.0	5	20.0	2	8.0	
			growth control	5	5	100.0	5	100.0	5	100.0	

Annex 5: Detailed results of BPGV, continued

B) Experiment II – Different methods, continued

				1. Evaluation 2 weeks after rewarming			**2. Evaluation** 4 weeks after rewarming		**3. Evaluation** 10 weeks after rewarming		
Acc. No.	Collecting date	experiment date	treatment	No. of explants	No. of green explants	Survival rate 1 (%) on 30.07.2010	No. of green explants	Survival rate 2 (%) on 16.08.2010	No of plantlets	Regrowth rate (%) on 27.09.2010	
6902 / I	28.06.10	19/20.07.2010	- LN	20	17	85.0	18	90.0	18	90.0	**droplet-vitrification**
			+ LN	25	16	64.0	21	84.0	19	76.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
6902 / II			- LN	20	16	80.0	20	100.0	18	90.0	
			+ LN	25	5	20.0	18	72.0	12	48.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
6902 / I			- LN	20	7	35.0	19	95.0	17	85.0	**vitrification**
			+ LN	25	2	8.0	6	24.0	3	12.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
6902 / II			- LN	20	8	40.0	19	95.0	18	90.0	
			+ LN	25	0	0.0	2	8.0	0	0.0	
			growth control	5	5	100.0	5	100.0	5	100.0	

Annex 5: Detailed results of BPGV, continued

C) Experiment III – Different inflorescence stages

				1. Evaluation 2 weeks after rewarming			**2. Evaluation** 4 weeks after rewarming		**3. Evaluation** 10 weeks after rewarming		
Acc. No.	Collecting date	experiment date	treatment	No. of explants	No. of green explants	Survival rate 1 (%) on 23.07.2010	No. of green explants	Survival rate 2 (%) on 16.08.2010	No. of plantlets	Regrowth rate (%) on 16.09.2010	
171 K / I	10.05.10	05./06.07.2010	- LN	20	4	20.0	12	60.0	10	50.0	**stage A**
			+ LN	25	9	36.0	11	44.0	9	36.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
171 K / II			- LN	20	12	60.0	16	80.0	12	60.0	
			+ LN	25	7	28.0	8	32.0	6	24.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
171 K / I	17.05.10		- LN	20	10	50.0	18	90.0	16	80.0	**stage K**
			+ LN	25	15	60.0	21	84.0	17	68.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
171 K / II			- LN	20	17	85.0	20	100.0	18	90.0	
			+ LN	25	17	68.0	19	76.0	19	76.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
171 K / I	04.06.10		- LN	20	18	90.0	19	95.0	18	90.0	**stage O**
			+ LN	25	16	64.0	24	96.0	20	80.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
171 K / II			- LN	20	14	70.0	18	90.0	16	80.0	
			+ LN	25	13	52.0	20	80.0	20	80.0	
			growth control	5	5	100.0	5	100.0	5	100.0	

Annex 5: Detailed results of BPGV, continued

D) Experiment IV – Different storage durations

				1. Evaluation 2 weeks after rewarming				**2. Evaluation** 4 weeks after rewarming			**3. Evaluation** 10 weeks after rewarming		
Acc. No.	Collecting date	experiment date	treatment	No. of explants	No. of green explants	Survival rate 1 (%)	Date of observation	No. of green explants	Survival rate 2 (%)	Date of obser-vation	No. of plantlets	Regrowth rate (%) on 27.09.2010	
350 K / I	11.06.10	14./15.06.2010	- LN	20	17	85.0	02.07.10	16	80.0	16.07.10	13	65.0	**Only 0 - 2 days**
			+ LN	25	9	36.0		9	36.0		5	20.0	
			growth control	5	5	100.0		5	100.0		5	100.0	
350 K / II			- LN	20	17	85.0		18	90.0		16	80.0	
			+ LN	25	6	24.0		6	24.0		4	16.0	
			growth control	5	5	100.0		5	100.0		5	100.0	
350 K / I		12./13.07.2010	- LN	20	20	100.0	30.07.10	20	100.0	16.08.10	12	60.0	**4 weeks**
			+ LN	25	14	56.0		14	56.0		11	44.0	
			growth control I	5	5	100.0		5	100.0		5	100.0	
350 K / II			- LN	20	15	75.0		15	75.0		13	65.0	
			+ LN	25	16	64.0		15	60.0		7	28.0	
			growth control	5	5	100.0		5	100.0		5	100.0	
350 K / I		26./27.07.2010	- LN	20	16	80.0	16.08.10	16	80.0	30.08.10	14	70.0	**6 weeks**
			+ LN	25	12	48.0		13	52.0		8	32.0	
			growth control	5	5	100.0		5	100.0		4	80.0	
350 K / II			- LN	20	14	70.0		14	70.0		8	40.0	
			+ LN	25	17	68.0		17	68.0		6	24.0	
			growth control	5	5	100.0		5	100.0		5	100.0	

Annex 5: Detailed results of BPGV, continued

E) Experiment V – Different incubation times of PVS 3

				1. Evaluation 2 weeks after rewarming			**2. Evaluation** 4 weeks after rewarming		**3. Evaluation** 10 weeks after rewarming		
Acc. No.	Collecting date	experiment date	treatment	No. of explants	No. of green explants	Survival rate 1 (%) on 30.07.10	No. of green explants	Survival rate 2 (%) on 16.08.10	No. of plantlets	Regrowth rate (%) on 20.09.2010	
348 K / I	10.05.10	13.07.10	- LN	20	18	90.0	18	90.0	9	45.0	**0.5 h**
			+ LN	25	12	48.0	12	48.0	7	28.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
348 K / II			- LN	20	19	95.0	19	95.0	11	55.0	
			+ LN	25	9	36.0	8	32.0	5	20.0	
			growth control	5	5	100.0	5	100.0	3	60.0	
348 K / I			- LN	20	15	75.0	14	70.0	11	55.0	**1.5 h**
			+ LN	25	14	56.0	14	56.0	11	44.0	
			growth control	5	5	100.0	5	100.0	4	80.0	
348 K / II			- LN	20	17	85.0	15	75.0	12	60.0	
			+ LN	25	13	52.0	13	52.0	9	36.0	
			growth control	5	5	100.0	5	100.0	4	80.0	
348 K / I			- LN	20	14	70.0	14	70.0	14	70.0	**2.5 h**
			+ LN	25	12	48.0	12	48.0	11	44.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
348 K / II			- LN	20	15	75.0	15	75.0	14	70.0	
			+ LN	25	10	40.0	12	48.0	13	52.0	
			growth control	5	5	100.0	5	100.0	5	100.0	

Annex 5: Detailed results of BPGV, continued

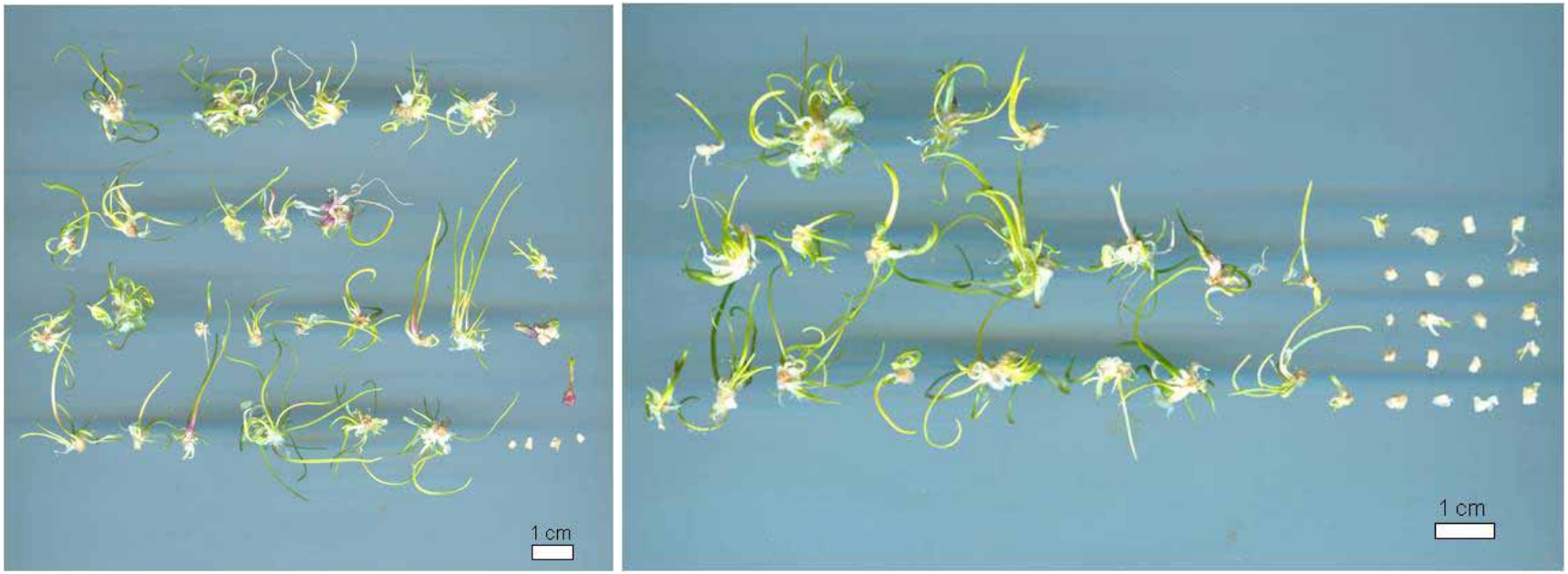
F) Experiment VI – Different PVS compositions

				1. Evaluation 2 weeks after rewarming			**2. Evaluation** 4 weeks after rewarming		**3. Evaluation** 10 weeks after rewarming		
Acc. No.	Collecting date	experiment date	treatment	No. of explants	No. of green explants	Survival rate 1 (%) on 16.08.10	No. of green explants	Survival rate 2 (%) on 30.08.10	No. of plantlets	Regrowth rate (%) on 27.09.2010	
7123 / I	11.06.10	26./27.07.2010	- LN	20	12	60.0	11	55.0	11	55.0	**PVS2**
			+ LN	25	14	56.0	11	44.0	8	32.0	
			growth control	5	4	80.0	4	80.0	4	80.0	
7124 / II			- LN	20	16	80.0	16	80.0	14	70.0	
			+ LN	25	21	84.0	C	-	C	-	
			growth control	5	5	100.0	C	-	C	-	
7123 / I			- LN	20	18	90.0	C	-	C	-	**PVS3**
			+ LN	25	C	-	C	-	C	-	
			growth control	5	C	-	C	-	C	-	
7124 / II			- LN	20	18	90.0	19	95.0	16	80.0	
			+ LN	25	19	76.0	17	68.0	12	48.0	
			growth control	5	5	100.0	5	100.0	5	100.0	
7123 / I			- LN	20	C	-	C	-	C	-	**PVS4**
			+ LN	25	C	-	C	-	C	-	
			growth control	5	C	-	C	-	C	-	
7124 / II			- LN	20	15	75.0	15	75.0	12	60.0	
			+ LN	25	0	0.0	0	0.0	0	0.0	
			growth control	5	5	100.0	C	-	C	-	

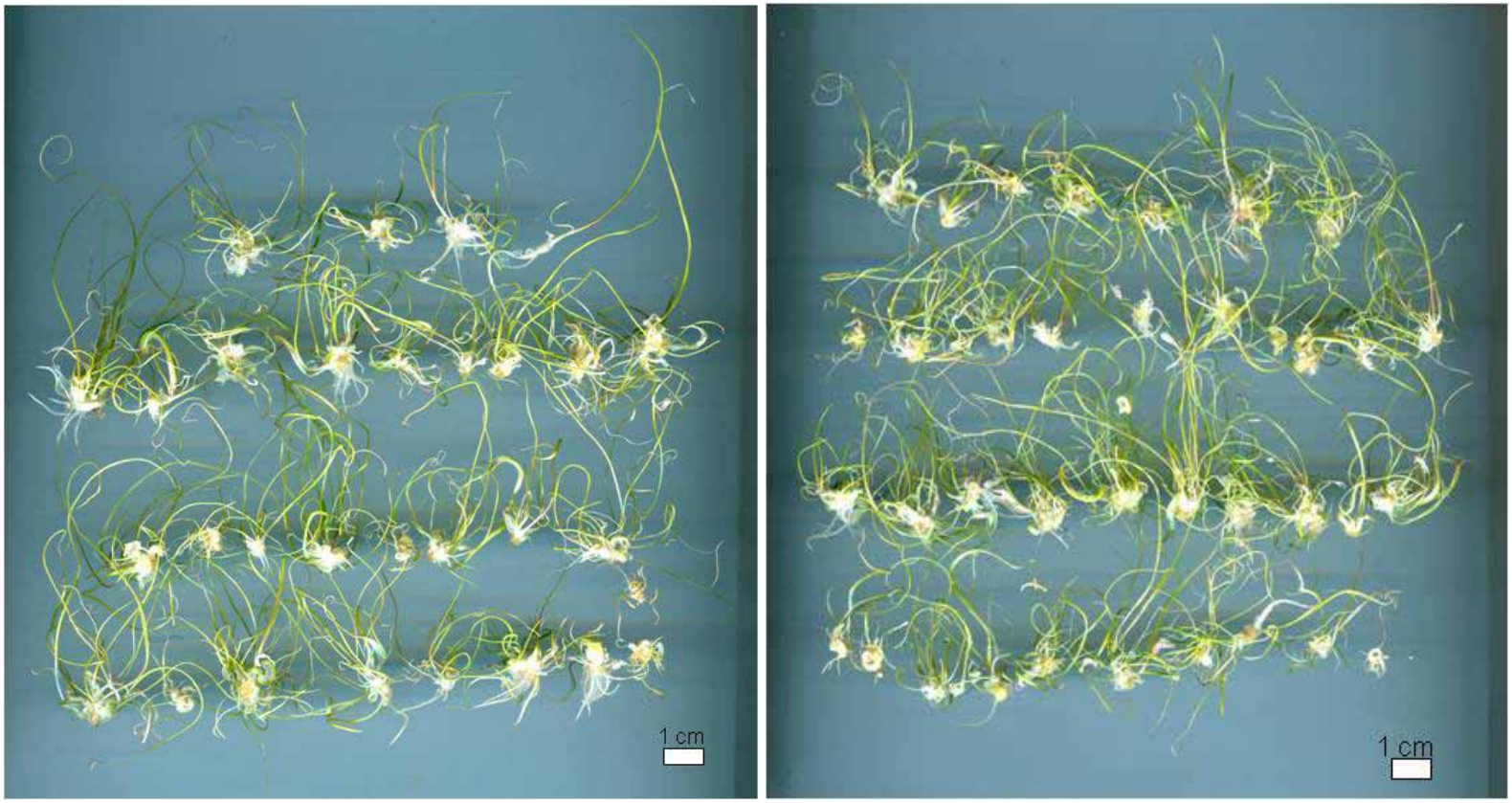
C = contaminations therefore no survival or regrowth rates

Annex 6: Pictures of the regenerated explants after rewarming

All 0232 regrowth 10 weeks after rewarming, left site: - LN; right site: +LN



348K regrowth 10 weeks after rewarming, left site: - LN; right site: +LN



7817 regrowth 10 weeks after rewarming, left site: - LN; right site: +LN