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In vitro regeneration in *Allium species*

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ABSTRACT

An attempt to induce shoot regeneration from leaf disc explants from *Allium sativum L.*, *A. porrum L.*, and *A. schoenoprasum L.* and the induction of shoot regeneration from single flower-bud receptacles in *A. porrum* is presented. While the regeneration rate from leaf disc explants was low, an efficient method for propagating *A. porrum in vitro* was obtained by cultivating single flower-bud receptacles. The shoot regeneration ability was strongly controlled by the genotype. Up to 294 shoots per leek plant could be harvested. Simultaneously the same plant could be used for seed production and bulbil formation *in vivo*. The efficiency of the *in vitro* multiplication method described allows the integration of this procedure into breeding programmes of *A. porrum*

ABBREVIATIONS

BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxy acetic acid; IAA, 3-indole acetic acid; NAA, 2-naphtalene acetic acid

INTRODUCTION

Vegetative propagation *in vitro* could be useful in breeding *Allium* species.

In leek (*Allium porrum L.*) the development of hybrid varieties is of great economical importance. Unfortunately, there is a strong inbreeding depression already in early generations (Schweisguth and Burant 1970), which is the bottleneck when producing seeds from selected hybrid-parent lines. Using *in vitro* culture for multiplication of hybrid-parent lines this disadvantage could be reduced. As the "conventional" hybrid breeding in *Allium* is based on a cytoplasmic male sterility system, maintainers have to be developed to reproduce the male sterile hybrid parent. Using an existing recessive genic male sterility in *Allium porrum* (Schweisguth and Burant 1970) the maintainer could be replaced by an "unconventional" *in vitro* multiplication of the male sterile parent. In addition *in vitro* storage of potential hybrid parents during their evaluation could reduce the developmental costs of the hybrid varieties.

Nearly all genotypes of garlic (*Allium sativum L.*) are sterile (Etho 1985). Thus breeding in this species is restricted to the selection of spontaneous mutants and their vegetative propagation *in vivo*. *In vitro* culture would allow efficient mutation induction and the mass propagation of selected genotypes.

In chive (*Allium schoenoprasum L.*) *in vitro* culture could be used for rapid multiplication of selected parent lines to produce seed of the population variety type. In addition planting material of homogeneous clone varieties could be raised by *in vitro* culture.

Existing *in vitro* multiplication techniques in *Allium* species are summarized in Novak *et al.* (1986). The efficiency of the methods available is mostly low with regard to the practical application. We therefore report in this paper attempts to induce shoot regeneration using leaf and receptacle explants.

MATERIALS AND METHODS

Plant material

Leek seedlings of the cvs. 'Winterreuzen Ator', 'Tropita', and breeding material kindly supplied by Dr. B. Schweisguth, Versailles, were grown in the greenhouse and in the field. Vernalization occurred naturally in the field during overwintering and was induced at +5°C in the winter period in the greenhouse.

Garlic cloves of unknown varieties originating from France, Italy and Yugoslavia, were cultivated in the greenhouse.

Chive seedlings of breeding material were raised *in vitro* on a LS-basic medium (Linsmaier and Skoog 1965) without growth regulators.

Explants and surface sterilization

The leaves of three month old greenhouse grown plants of leek and garlic were cut off about 10 cm above the plant base and rejected. After removing the stem, the remaining leaf pieces were separated from each other and surface sterilized for 5 min in a sodium hypochlorite solution (2.6% of active chlorine) followed by a 10 min wash with autoclaved water. The chive leaves *in vitro* harvested were not surface sterilized.

Surface sterilized leaves of leek and garlic were cut into discs of about 5 mm x 5 mm, chive leaves were cut into pieces of

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about 5 mm length. The leaf discs or leaf pieces were placed with the leaf face onto the medium.

Individual flower buds of leek were harvested from just opened umbels with single flowers mostly being closed. The flower buds were surface sterilized as described for the leaves, and the petals, stamens and the ovary removed. The remaining receptacle explants were put with their cut surface onto the culture medium.

Culture media and growing conditions

The explants were cultivated on solid LS-basic medium with 2 mmol·l^{-1} NH_4NO_3, 3% sucrose and 0.8% Agar (Serva No. 11396). The pH of the medium was adjusted to 5.8 prior to autoclaving.

Leaf discs: Twelve media variants with different growth regulator combinations designated as M1-M12 (Table 1) were used.

Flower-bud receptacles: The culture medium for flower-bud receptacles was supplemented with 0.011 mmol·l^{-1} NAA and 0.008 mmol·l^{-1} BAP. After four weeks the explants were transferred to a medium containing 0.005 mmol·l^{-1} NAA and 0.013 mmol·l^{-1} BAP.

All explants were grown in petri dishes at a temperature of 26°C with a photoperiod of 12 hours with about 3,000 lux from Osram-L 65 W/30 warm white lamps. Every four weeks the explants were subcultured on fresh medium.

Table 1: Culture media for leaf disc explants from *Allium porrum* (leek), *A. sativum* (garlic) and *A. schoenoprasum* (chive). A LS-basic medium with 2 mmol·l^{-1} NH_4NO_3 was complemented with auxins and cytokinins.

Culture media	Auxin [mmol·l^{-1}]			Cytokinin [mmol·l^{-1}]	
	NAA	IAA	2,4-D	BAP	Kinetin
M1	0.003	0.003	—	0.004	—
M2	0.003	0.011	—	0.004	—
M3	0.005	0.003	—	0.004	—
M4	0.005	0.011	—	0.004	—
M5	0.003	0.003	—	—	0.005
M6	0.003	0.011	—	—	0.005
M7	0.005	0.003	—	—	0.005
M8	0.005	0.011	—	—	0.005
M9	0.003	—	0.005	0.004	—
M10	0.005	—	0.005	0.004	—
M11	0.003	—	0.005	—	0.005
M12	0.005	—	0.005	—	0.005

RESULTS

Response of leaf disc explants

The contamination in leaf discs was about 9% in leek and up to 60% in garlic, whereas in chive no contamination occurred.

Response of leaf disc explants on culture media with and without 2,4-D is presented in Table 2.

Explants cultivated on media without 2,4-D generally showed a higher percentage of "enlargement" and a lower frequency of "white callus formation" and "no reaction".

Table 2: Influence of 2,4-D on leaf disc explants from *Allium porrum, A. sativum* and *A. schoenoprasum*, M1-M8 without, M9-M12 with 2,4-D.

Allium species	Culture media [a]	Number of explants	Response (% of explants)		
			Enlargement	White callus formation	No reaction
Allium porrum	M1-M8	1353	77.8	7.7	14.5
	M9-M12	643	47.2	29.0	23.8
Allium sativum	M1-M8	535	84.3	11.4	4.1
	M9-M12	256	40.6	47.3	12.1
Allium schoeno-prasum	M1-M8	947	28.7	1.7	69.6
	M9-M12	483	23.6	4.7	71.7

[a] see Table 1

There was no influence of the amount of NAA and IAA on explant response. However, a higher frequency of "white callus" was found in garlic explants on media with BAP instead of Kinetin.

Shoot regeneration from leaf disc explants began with the fourth week in garlic and chive and the sixth week in leek. The percentage of regenerating explants was 0.5% in leek, 1.3% in garlic, and 1.8% in chive. The regeneration was independent of the media used, and also of the colour and growth behaviour of the explants, but was strongly controlled by the genotype of the explant source. One to five plantlets per explant could be observed. The plantlets were regenerated from callus or directly from the leaf disc explant.

For rooting, shoots of about 5 cm of length were harvested and put on LS-basic medium without growth regulators. Differences in rooting ability between the *Allium* species used could be observed. While chive shoots rooted after only four to six days, and leek shoots after two weeks, garlic shoots needed between three and four weeks. All shoots could be successfully transferred to a soil and peat substrate in the greenhouse. They developed into normal plants.

Response of single flower bud receptacles of leek

Beginning with the tenth day after incubation direct shoot regeneration from single flower-bud receptacles could be observed.

In a first experiment, 92 genotypes of the variety 'Winterreuzen Ator' were tested. In eight genotypes (Table 3) regeneration occurred between 2% to 20% of the explants cultivated. The total number of isolated shoots varied between one in genotype A5 and A6 and 234 in genotype A8 (Table 3). The mean number of shoots produced per explant is about 8.6 in the genotype A8. The shoots were rooted as described, and developed into normal plants in soil in the greenhouse.

In a second experiment, 28 genotypes of the French leek material, 37 genotypes of the cv. 'Winterreuzen Ator', and 25 of the cv. 'Tropita' were tested.

Table 3: Shoot regeneration in leek (cv. 'Winterreuzen Ator') from single flower-bud receptacles grown on LS-basic medium with 2 mmol·l^{-1} NH_4NO_3 and 0.011 mmol·l^{-1} NAA and 0.008 mmol·l^{-1} BAP, first experiment.

Genotype	Explants		No. harvested shoots	No. shoots transferred to soil [a]
	no. cultivated	% with shoots		
A 1	57	14.0	24	15
A 2	44	9.0	29	36
A 3	41	12.2	3	14
A 4	38	18.4	10	6
A 5	25	2.0	1	—
A 6	25	2.0	1	—
A 7	30	3.3	4	—
A 8	50	20.0	234	235

[a] In few cases shoots showed multiple shoot formation during rooting

In the French material 22 genotypes were able to regenerate shoots from single flower-bud receptacles (Table 4). From 17 genotypes shoots were harvested for rooting and 12 genotypes could be transferred to soil in the greenhouse. From the other genotypes with regeneration ability no shoots could be harvested due to infections.

Within the varieties 'Winterreuzen Ator' and 'Tropita' (Table 5) 16 of the genotypes tested were able to regenerate shoots. From 11 genotypes shoots could be harvested for rooting and 9 genotypes could be transferred to soil in the greenhouse. The shoot development was normal.

DISCUSSION

In leek Debergh and Standaert-de Metsenaere (1976) as well as Dunstan and Short (1979) induced bulbil formation from callus or directly from explants of the basal plate. Dunstan and Short (1979) harvested an average of up to 120 shoots per leek plant. Shoot regeneration from pieces of flower-head receptacles were obtained by Novak and Havel (1981). Using the base of the inflorescence Doré and Schweisguth (1980) obtained 10 to 80 plantlets per inflorescence.

In garlic Havranek and Novak (1973), Novak and Havranek (1974) and Novak (1980) used basal parts of leaf blades as explants and induced bud formation from callus of these explants. Novak (1980) obtained a total of 808 plants, but did not describe the number of explants cultivated. Shoot formation out of shoot tips was found by Novak (1983), Bhojwani *et al.* (1982/83) and Abo el Nil (1976), who also induced shoot differentiation from callus produced on bulb-leaf explants and stem segments. Callus induction and plantlet regeneration from storage leaves of garlic were achieved by Zhou *et al.* (1980), while Lü *et al.* (1982) reported plantlet differentiation in callus induced from young leaves. Bhojwani (1980) used "shoot bud" explants from dormant bulbs and obtained shoot proliferation.

Table 4: Shoot regeneration in leek (French material) from single flower-bud receptacles grown on LS-basic medium with 2 mmol·l^{-1} NH_4NO_3 and 0.011 mmol·l^{-1} NAA plus 0.008 mmol·l^{-1} BAP, second experiment.

Genotype	Explants		Mean no. of harvested shoots per regenerating explant and harvesting date					Total no. of of harvested shoots	Max. no. of harvested shoots/ explant	No. of shoots transferred to soil
	no. cultivated	% with shoots	1	2	3	4	5			
P5/116	60	1.6	2.0	—	—	—	—	2	2.0	9
P5/ 16	82	69.5	—	—	—	—	—	—	—	—
P5/ 66	25	4.0	1.0	—	—	—	—	1	1.0	1
P5/ 27	32	12.5	1.0	1.0	—	—	—	2	1.0	2
P5/ 71	25	12.0	1.0	2.0	5.0	—	—	17	9.0	17
P5/114	25	20.0	—	—	—	—	—	—	—	—
P5/ 91	25	36.0	1.0	3.6	—	—	—	13	8.5	12
P5/ 96	25	12.0	1.0	4.5	1.0	1.0	—	14	10.0	10
P5/107	25	12.0	—	—	—	—	—	—	—	—
P5/ 90	25	12.0	2.0	—	—	—	—	6	2.5	6
P5/ 78	25	4.0	2.0	—	—	—	—	2	2.0	—
P5/ 83	25	20.0	1.0	1.0	—	—	—	4	3.0	—
P5/ 65	25	32.0	2.5	2.5	0.3	—	—	21	6.5	16
P5/110	25	8.0	1.0	—	—	—	—	4	1.0	6
P5/105	25	12.0	—[b]	—	—	—	—	11	—	—
P5/ 28	25	56.0	3.5	6.0	—	—	—	13	6.0	16
P5/131	25	4.0	—	—	—	—	—	—	—	—
P5/119	25	44.0	—	—	—	—	—	—	—	—
P5/108	25	8.0	—	—	—	—	—	—	—	—
P6/ 1[a]	72	62.5	2.2	2.5	3.1	4.9	5.8	295	39.0	208
P6/ 5	25	4.0	—	—	—	—	—	—	—	—
P4/ 1	25	20.0	—[b]	—	—	—	—	3	—	3

[a] Shoot harvesting in this genotype is still continuing. [b] Figures not collected.
The genotypes P5/ 16, P5/114, P5/107, P5/131, P5/119, P5/108, and P6/ 5 failed after infections.

Table 5: Shoot regeneration in leek (cvs. 'Winterreuzen Ator' and 'Tropita') from single flower-bud receptacles grown on LS-basic medium with 2 mmol·l^{-1} NH_4NO_3 and 0.011 mmol·l^{-1} NAA plus 0,008 mmol·l^{-1} BAP, second experiment.

Genotype[a]	Explants		Mean no. of harvested shoots per regenerating explant and harvesting date			Total no. of harvested shoots	Max. no. of harvested shoots/ explant	No. shoots transferred to soil
	no. cultivated	% with shoots	1	2	3			
T12/46	50	2.0	—	—	—	—	—	—
T12/45	50	2.0	4.0	—	—	4	4.0	4
T12/51	25	36.0	2.1	1.0	—	20	4.0	10
A 8	25	4.0	5.0	7.0	—	13	7.0	5
A1/510	25	4.0	—	—	—	—	—	—
A0/ 61	25	4.0	—	—	—	—	—	—
T92/ 4	25	4.0	3.0	—	—	3	3.0	4
T72/55	25	24.0	—	1.8	2.5	21	4.0	6
T92/38	25	4.0	—	—	—	—	—	—
T92/ 2	25	8.0	1.0	0.5	—	3	1.0	3
A03/ 4	25	4.0	—	2.0	—	2	2.0	—
A04/15	25	8.0	—	—	—	—	—	—
A13/ 7	25	4.0	1.0	—	—	1	1.0	1
A03/19	25	8.0	—[b]	—	—	9	—	4
A20/27	25	4.0	—[b]	—	—	1	—	—
A54/ 1	25	4.0	5.0	2.0	—	7	5.0	5

[a]A = 'Winterreuzen Ator', T = 'Tropita'; [b]Figures not collected.
The genotypes T12/46, A1/510, A0/61, T92/38, and A04/15 failed after infections.

The frequency of regenerative explants and the number of plantlets harvested was too low in most cases to integrate *in vitro* methods in practical breeding of *Allium*.

Shoot regeneration from leaf discs, as reported in this paper, was as low as 0.5% of explants in leek, 1.3% in garlic and 1.8% in chive. Therefore, this *in vitro* method seems to be of no value to be integrated in *Allium* breeding programmes. However, this method may be applied to conserve non flowering breeding material.

As demonstrated in *Brassica*, single flower-bud receptacles are very regenerative explants (Dunemann and Grunewaldt 1987). Also in leek flower-bud receptacle explants regenerated shoots (Tables 3, 4, and 5). The total number of harvested shoots varies between 1 and 295. The shoot harvesting is still going on, growing the explants on the shooting medium with 0.005 mmol·l^{-1} NAA and 0.013 mmol·l^{-1} BAP.

Although a genotypic variability for regeneration capacity was found, *in vitro* vegetative propagation from receptacle explants is very efficient. The number of shoots obtained allows the integration of this procedure into a hybrid breeding programme of leek. If economic, *in vitro* propagation could be used to produce planting material of the male sterile and, if necessary, also of the male fertile hybrid parent.

Another advantage of the *in vitro* propagation of leek described is that it is not necessary to destroy the plant or to take the whole inflorescence as explant as proposed by Dunstan and Short (1979) and Doré and Schweisguth (1980). Thus, an individual genotype can be evaluated during its vegetative and generative development. It can be used as explant source and for seed production. Simultaneously bulbils can be harvested to maintain the selected genotype *in vivo*.

Nevertheless, an efficient *in vitro* regeneration system is a prerequisite for the application of other *in vitro* techniques: especially the induction of male sterility, the selection of resistance, and particularly the storage of breeding material of *Allium* species.

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