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Efficient *in vitro* regeneration of leek (*Allium ampeloprasum* L.) via flower stalk segments

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Summary. A new simple, efficient and rapid *in vitro* method for mass clonal propagation of leek (*Allium ampeloprasum* L.) plants, using small (5 mm) flower stalk (peduncle) explants, was established. Adventitious shoots were produced from single subepidermal cells. A wide variation in the percentage of regenerating explants and number of regenerated shoots per explant between individual plants within one cultivar was observed. The concentration of the growth regulators 6-benzylaminopurine and α-naphthalene-acetic acid influenced the percentage of regenerating explants and the average number of regenerated shoots per explant. A combination of 10 mg.l^{-1} 6-benzylaminopurine and 10 mg.l^{-1} α-naphthalene-acetic acid, resulted in a maximum percentage of regenerating explants and a high average number of regenerated shoots per explant. The percentage of regenerating explants and the average number of regenerated shoots per explant decreased with increasing flower stalk length (age). The basal explants gave both the highest percentage of regenerating explants and average number of regenerated shoots per explant. An average of 300 shoots per flower stalk was obtained for all plants, making this new *in vitro* method a powerful tool in hybrid leek breeding.

Abbreviations: BAP - 6-benzylaminopurine, NAA - α-naphthalene-acetic acid, MS - Murashige and Skoog (1962) medium, PREX - percentage of regenerating explants, NSHO - average number of regenerated shoots per explant.

Introduction

Leek (*Allium ampeloprasum* L.) is an autotetraploid (2n=4x=32) mainly outbreeding and monocotyledonous crop, grown for its edible (false) stem. Present leek cultivars are open pollinated populations and, hence, heterogeneous and unstable.

The most promising system for improvement of uniformity, yield and disease resistance seems to be hybrid breeding. Hybrid leek breeding is hampered by the lack of a suitable system for production of male sterile genotypes. In leek, much effort is being undertaken to create a genetic system based on (cytoplasmic) male sterility by using protoplast, transformation and mutation techniques. However, successful application of these methods depends on the availability of an efficient *in vitro* regeneration and propagation method.

In vitro multiplication via organogenesis and somatic embryogenesis, has been described for leek (for a review see Novák *et al.* 1986). Stem explants, basal plates, leaf discs, flower heads, open flowers, single flower bud receptacles and anthers have been used as explant material (Debergh and Standaert-de Metsenaere 1976; Dunstan and Short 1979; Doré and Schweisguth 1980; Novák and Havel 1981; Van Doorne *et al.* 1988; Rauber and Grunewaldt 1988; Doré 1988; Baumunk-Wende 1989). Somatic embryogenesis in leek has been described by Van der Valk *et al.* (1992) and Buiteveld *et al.* (1993).

Regeneration from explants of the flower stalk (peduncle) has been described for species belonging to the *Amaryllidaceae* family (*Nerine*, *Eucharis* and *Hippeastrum*) by Pierik *et al.* (1985), Koopman *et al.* (1987) and De Bruyn *et al.* (1992). Ziv *et al.* (1983) briefly reported shoot development on explants from young elongating inflorescence stalks of ornamental forms of *Allium ampeloprasum* L.

The general aim of this study was to establish an efficient regeneration method using flower stalk segments and to optimize medium and culture conditions for the benefit of large scale *in vitro* multiplication of genotypically different leek plants. An efficient *in vitro* regeneration method must be applicable for all genotypes and a high multiplication factor per genotype must be realized.

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Materials and Methods

Plant material. Experiments were performed with mature leek plants of the cultivar **Vrizo** and *in vitro* cloned plants of different genotypes (**Por-1**, **Por-2**, **Por-3**, **Por-4**, **Por-256**, **Por-266**, **Por-286**, **Por-328** and **Por-334**), derived from the cultivar **Porino**. Both cultivars were bred by Nunhems Zaden (Haelen, The Netherlands). The *in vitro* cloned plants of cultivar **Porino** were obtained via somatic embryogenesis. All plants were grown in the field and, after natural vernalization during the winter period, transplanted in March 1993 to a greenhouse (20°C, 16/8 h photoperiod). Bolting started a few weeks after transfer to the greenhouse. Flower stalks (peduncles) and small parts of the basal plates were isolated from bolting plants, surface sterilized for 30 sec in 70% (v/v) ethanol and 20 min in 1.5% (v/v) sodium hypochlorite (commercial bleach) with a few drops of Tween 20 and subsequently rinsed several times (5, 10, 15 and 20 min) in sterile water. After sterilization 5 mm large discs were cut from the flower stalk and placed with the proximal side down on regeneration medium.

Media and Culture conditions. The standard medium contained MS salts and vitamins (Murashige and Skoog 1962), 30 $g.l^{-1}$ sucrose, 8 $g.l^{-1}$ agar (Daishin, Brunschwig) and 200 $mg.l^{-1}$ cefotaxime sodium (Duchefa, Haarlem). The growth regulators BAP and NAA were added to the media in different concentrations and the pH was adjusted to 5.8 with 1 M KOH prior to autoclaving for 20 min. Cultures were incubated in glass jars at 21°C under 12/12 h photoperiod (approx.20 $W.m^{-2}$) and subcultured at three week intervals. After nine weeks, shoots were placed on half strength MS medium without growth regulators for further rooting. After another three weeks, young plantlets were separated individually and transferred to soil, where they were kept at a high humidity for one to two weeks.

Experiments and Statistical Analysis. After a period of 9 weeks the number of regenerating explants and the number of regenerated shoots per explant was counted, although shoot regeneration continued for a longer period. Only shoots measuring more than 5 mm were counted and classified. The percentage of regenerating explants per treatment was recorded as PREX and the average number of regenerated shoots per explant was calculated and recorded as NSHO. No normal distribution of the data was found in our experiments. Therefore, results were analysed using the Mann-Whitney 'U' test with $P \leq 0.05$.

Histology. For histological studies, tissues were fixed in 5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. The fixed tissues were rinsed twice with phosphate buffer for 1 h and water for 30 min. After dehydration through a graded alcohol series, the tissues were embedded in Historesin. Sections of 8 μm thickness were cut with a rotation microtome (Reichert-Jung), stained with toluidine blue (Feder and O'Brian 1968) and examined with a light microscope.

Results

1. Genotypic effects on plant regeneration.

The genotypic effect on the percentage of regenerating explants (PREX) and the average number of regenerated shoots (NSHO) was assessed per genotype, by using explant material from 29 genotypically different plants of the cultivar **Vrizo**. These plants showed differences in flower stalk length and, hence, these lengths were noted as a variable factor. Explants were cut from the basal part of flower stalks and placed on standard medium containing 1 $mg.l^{-1}$ BAP and 1 $mg.l^{-1}$ NAA.

Significant differences ($P \leq 0.05$) in PREX and NSHO were found between the 29 plants (Table 1). The PREX varied between 10 and 100 percent. An overall PREX of 69% for all plants was found. All plants cultured on the standard medium regenerated shoots. Between 0 and 160 shoots were harvested per explant. The NSHO varied between 0 and 40 for the different plants. An overall average of 12 shoots per explant per plant was found. Plants with the same flower stalk length showed significant differences in both PREX and NSHO. For example, the PREX and the NSHO for the five plants with flower stalk lengths of six centimeter varied from respectively 40% to 100% and from 3 to 36.

Significant ($P \leq 0.05$) negative correlation and regression coefficients were found between flower stalk length and PREX (resp. -0.13 and -0.46) and between flower stalk length and NSHO (resp. -0.13 and -0.21). A relatively long flower stalk resulted in a reduced PREX and NSHO for all plants cultured.

Table 1. Percentage of regenerating explants (PREX) and average number of regenerated shoots per explant (NSHO) for 29 genotypically different plants of cultivar **Vrizo** with varying flower stalk lengths and 8-30 explants per flower stalk.

Plant	Length[1]	PREX	NSHO
1	4	100 c[3]	16 c
2	5	100 c	40 e
3	5	66 bc	9 bc
4	6	100 c	18 c
5	6	100 c	17 c
6	6	40 ab	3 ab
7	6	50 ab	6 b
8	6	100 c	36 d
9	7	46 ab	5 bc
10	8	58 bc	5 bc
11	9	75 bc	4 bc
12	9	70 bc	7 bc
13	10	50 ab	7 bc
14	11	91 c	17 c
15	13	100 c	24 c
16	13	68 bc	21 c
17	14	94 c	21 c
18	15	76 bc	7 bc
19	17	46 ab	7 bc
20	17	40 ab	3 ab
21	19	76 bc	22 bc
22	19	80 bc	12 bc
23	26	50 ab	11 bc
24	26	63 bc	11 bc
25	35	28 ab	4 ab
26	43	85 c	9 bc
27	43	86 c	12 bc
28	46	40 ab	2 ab
29	47	10 a	0 a
TAV[2]		69	12

[1] Length of the flower stalk in cm

[2] Total average

[3] Within each column treatments with different letters are significantly different at the 5% level(Mann-Whitney test).

2. Medium and physiological effects on plant regeneration.

2.1. Effect of flower stalk length and concentration of growth regulators on plant regeneration.

The effect of BAP and NAA on shoot regeneration was studied for two cloned plants (genotypes). The flower stalk lengths were noted as variable factor. Explants originating from elongating flower stalks of clone **Por-286** and **Por-328**, were placed at random on media containing different combinations of the growth regulators BAP and NAA.

Both percentage of regenerating explants (PREX) and average number of regenerated shoots per explant (NSHO) increased significantly with a higher NAA concentration for both genotypes (Table 2a and 2b). A concentration of 0.1 mg.l^{-1} NAA resulted in both the lowest PREX and NSHO, whereas a combination of 10 mg.l^{-1} BAP and 10 mg.l^{-1} NAA resulted in both the highest PREX and NSHO. Similar to the experiments with cultivar **Vrizo**, differences were found in PREX and NSHO between the two genotypes. **Por-286** showed the highest total PREX (84%) and total NSHO (26). The PREX and the NSHO for both genotypes were not influenced significantly, in this experiment, by the flower stalk length. A decrease in PREX and NSHO caused by an increase in flower stalk length was found only for genotype **Por-328** in combination with treatments of 0.1 mg.l^{-1} NAA. In the experiments with cultivar **Vrizo** we found that the PREX and the NSHO decreased with increased flower stalk length and that a flower stalk length of 10 cm resulted in an optimal NSHO. For **Por-286** we noticed, however, that a flower stalk length of 10 cm resulted in the lowest PREX (67%).

Table 2a. Effect of flower stalk length (cm) and concentration of the growth regulators BAP and NAA on the percentage of regenerating explants (PREX) and the average number of regenerated shoots per explant (NSHO) per treatment for clone **Por-328** of cultivar **Porino**. Per flower stalk 25-30 explants were obtained.

		BAP:NAA concentration in mg.l^{-1} [1]						
Length		1:0.1	10:0.1	1:1	10:1	1:10	10:10	TAV[2]
12	PREX	0 *a1*[3]	0 *a1*	67 *ab1*	100 *b1*	100 *b1*	100 *b1*	65 *1*
19	PREX	20 *ab1*	0 *a1*	40 *ab1*	60 *b1*	67 *b1*	80 *b1*	43 *1*
23	PREX	30 *a1*	20 *a1*	50 *ab1*	70 *b1*	67 *b1*	80 *b1*	53 *1*
35	PREX	20 *a1*	0 *a1*	40 *ab1*	60 *b1*	100 *b1*	80 *b1*	50 *1*
12	NSHO	0 *a1*	0 *a1*	8 *b1*	6 *b1*	16 *b1*	17 *b1*	8 *1*
19	NSHO	0 *a1*	0 *a1*	7 *a1*	9 *a1*	9 *a1*	16 *b1*	10 *1*
23	NSHO	0 *a1*	3 *a1*	9 *b1*	8 *b1*	16 *b1*	15 *b1*	10 *1*
35	NSHO	0 *a1*	0 *a1*	5 *a1*	4 *a1*	10 *b1*	9 *b1*	5 *1*
TAV	PREX	22 *a*	9 *a*	48 *b*	72 *bc*	81 *c*	83 *c*	52
	NSHO	0 *a*	1 *a*	7 *b*	7 *b*	14 *c*	14 *c*	9

Table 2b. Effect of flower stalk length (cm) and concentration of the growth regulators BAP and NAA on the percentage of regenerating explants (PREX) and the average number of regenerated shoots per explant (NSHO) per treatment for clone **Por-286** of cultivar **Porino**. Per flower stalk 25-30 explants were obtained.

		BAP:NAA concentration in mg.l^{-1} [1]						
Length		1:0.1	10:0.1	1:1	10:1	1:10	10:10	TAV[2]
9	PREX	100 *a1*[3]	100 *a1*	100 *a1*	100 *a1*	67 *a1*	100 *a1*	93 *1*
10	PREX	33 *a1*	33 *a2*	100 *a1*	66 *a1*	67 *a1*	100 *a1*	67 *2*
14	PREX	33 *ab1*	20 *b2*	100 *a1*	100 *a1*	100 *a1*	100 *a1*	78 *12*
20	PREX	40 *a1*	-	100 *b1*	100 *b1*	100 *b1*	100 *b1*	88 *1*
25	PREX	80 *a1*	60 *a12*	100 *a1*	100 *a1*	-	100 *a1*	88 *1*
9	NSHO	64 *b2*	19 *a2*	39 *b1*	30 *b2*	17 *a1*	43 *b12*	37 *2*
10	NSHO	14 *a12*	22 *a2*	25 *a1*	28 *a12*	19 *a1*	28 *a12*	21 *1*
14	NSHO	12 *b12*	1 *a1*	17 *b1*	7 *b1*	25 *c1*	27 *c1*	15 *1*
20	NSHO	6 *a1*	-	27 *b1*	29 *b12*	37 *c1*	50 *c2*	30 *2*
25	NSHO	3 *a1*	8 *a2*	29 *b1*	37 *b2*	-	43 *b12*	29 *2*
TAV	PREX	54 *a*	50 *a*	100 *b*	96 *b*	90 *b*	100 *b*	84
	NSHO	17 *a*	14 *a*	26 *b*	27 *bc*	29 *bc*	41 *c*	26

[1] Concentration of the growth regulators BAP and NAA; for example 1:0.1 NAA =1 mg.l^{-1} BAP and 0.1 mg.l^{-1} NAA

[2] Total average

[3] Within each row treatments with different letters are significantly different- and within each column treatments with different numbers are significantly different at the 5% level (Mann-Whitney test).

2.2. Effect of the explant position within the flower stalk on plant regeneration.

The length of the flower stalk is related to the physiological age and growth occurs in the basal region. Therefore, the position (origin) of the explants within the flower stalk on plant regeneration was studied, using four *in vitro* cloned plants (genotypes) (**Por-1**, **Por-2**, **Por-3** and **Por-4**) which possessed equally sized flower stalks and were cultured on medium containing 1 $mg.l^{-1}$ BAP and 1 $mg.l^{-1}$ NAA.

On average, no significant differences in percentage of regenerating explants (PREX) were found between basal and apical explants (Table 3). However, significant differences between the four genotypes in PREX were observed: **Por-1** showing the lowest and **Por-4** the highest PREX. In general, basal explants produced, in comparison with apical ones, significantly the highest average number of shoots per explant (NSHO) (Table 3). By contrast, apical explants from **Por-2** showed a higher NSHO than basal explants. Significant differences between the four genotypes in NSHO were found. **Por-1** gave the lowest and **Por-4** the highest NSHO.

Table 3. Effect of the position of the explant in the flower stalk on the percentage of regenerating explants (PREX) and the average number of regenerated shoots per explant (NSHO) per treatment for the clones **Por-1**, **Por-2**, **Por-3** and **Por-4** of cultivar **Porino**. Per flower stalk 25 explants were obtained.

Origin		Basal	Apical	TAV[1]
Clone				
Por-1	PREX	97 *a2*[2]	40 *b1*	68 *1*
Por-2	PREX	100 *a2*	95 *a1*	97 *3*
Por-3	PREX	67 *a1*	100 *b1*	83 *2*
Por-4	PREX	100 *a2*	100 *a1*	100 *3*
Por-1	NSHO	17 *a1*	3 *b1*	10 *1*
Por-2	NSHO	6 *a1*	23 *b2*	15 *2*
Por-3	NSHO	30 *a2*	15 *b12*	23 *3*
Por-4	NSHO	27 *a2*	30 *a2*	27 *3*
TAV	PREX	88 *a*	81 *a*	85
	NSHO	21 *b*	16 *a*	18

[1] Total average

[2] Within each row treatments with different letters are significantly different- and within each column treatments with different numbers are significantly different at the 5% level (Mann-Whitney test).

3. Morphological and Histological Observations.

Elongation of explants occurred within a few days after transfer to culture medium. After one week a two- to four-fold increase in original length was observed. After a few days swellings were observed at the basal part of the explants. Histological examination of the flower stalk explants revealed that after 5 days of culture individual subepidermal cells were undergoing active cell division (Fig.1). Internal segmenting divisions within single cells were the initial step of the regeneration process. Continued development of these meristematic regions resulted in the production of adventitious shoot primordia (Fig.2), without any indication of callus formation. Within four weeks individual shoots were clearly visible emanating as clusters from the base of the individual flower stalk segments. Root formation started after approximately six weeks of culture on the same medium and clusters of rooted plantlets were established after nine weeks. After this period, explants exhibited leaf like structures and, occasionally, single flower buds. The ratio between leaf like structures and shoots was 1:2. After another three weeks on half strength MS medium without growth regulators, young rooted plantlets were easily separated from the remaining explant and other plantlets. More than 90% of the rooted plantlets was successfully transferred to soil. No morphological deviations were observed in the field among 1400 mature cloned leek plants of several genotypes obtained via this method.

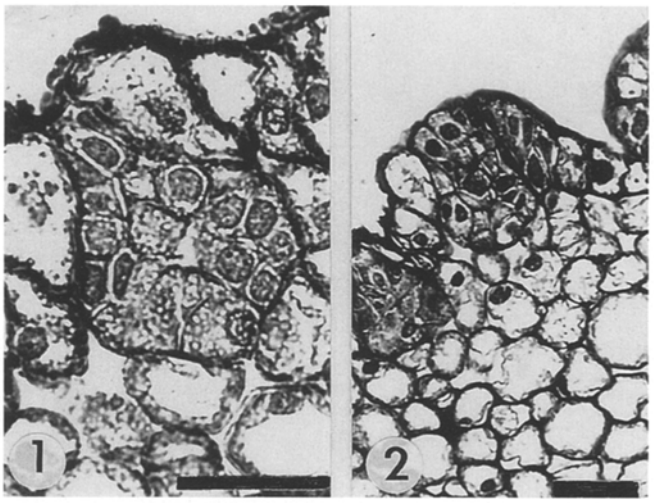

Fig.1. Transverse section near the basal cut end, showing active cell division within single subepidermal cells (Bar= 5μm).

Fig.2. Transverse section showing young shoot primordia emerging through epidermis (Bar=5μm).

Discussion

A new and high-frequency regeneration method for mass clonal propagation of leek plants, using flower stalk explants, was established. This *in vitro* propagation method offers possibilities for large scale micropropagation of selected leek plants for research and breeding purposes, and in particular for maintaining male sterile lines.

A genotypic effect on PREX and NSHO was found in our experiments among 29 genotypically different plants of leek cultivar **Vrizo**. However, the plants showed differences in flower stalk lengths and, therefore it was not possible to clearly distinguish between the effect of

the genotype and the effect of the flower stalk length on PREX and NSHO. The genotype is one of the most influential factors in determining the organogenic response (Brown and Thorpe 1986; Halperin 1986). Rauber and Grunewaldt (1988) also found that shoot regeneration from young single flower bud receptacles of leek was strongly controlled by the genotype. They obtained a maximum of 294 shoots per leek plant. Debergh and Standaert-de Metsenaere (1976) induced an average of three bulbils per explant on stem explants of leek and Dunstan and Short (1979) obtained an average of 120 shoots per leek plant on basal plate tissue. The inflorescence fragment culture described by Doré and Schweisguth (1980) and Novák and Havel (1981) gave a high shoot regeneration (10-80 shoots per inflorescence) and showed a genotypic influence as well. An average of 245 and a maximum of 800 shoots per flower stalk (approx. 10 cm) of cultivar **Vrizo** was obtained within nine to twelve weeks by our method. By contrast, Ziv *et al.* (1983) only obtained four shoots per inflorescence stalk.

For our method a low contamination rate (<3%) was found as compared to the earlier mentioned methods. However, a high bacterial contamination rate (38%) was observed in the experiment with cultivar **Vrizo**. This high contamination rate was caused by the fact that the bolting plants came directly from the field.

The PREX and NSHO were influenced by the concentration of the auxin NAA and the cytokinin BAP in the medium. This is in accordance with results from other species (Thorpe and Patel 1986). Koopman *et al.* (1987) found that a BAP:NAA ratio of 10:1 was optimal for shoot regeneration from flower stalk explants in *Amaryllis* species, whereas Ziv *et al.*(1983) obtained "good" shoot regeration with a 6:1 ratio in *Allium ampeloprasum* L.. In our experiments with leek, the NAA concentration seemed to be more crucial for regeneration than the BAP concentration. A relatively high NAA concentration resulted in an increased PREX and NSHO.

The length of the flower stalk and the origin of the explant influenced the PREX and NSHO. The length of the flower stalk is a measure of the physiological age. Relatively long and old flower stalks showed a reduced PREX and NSHO. Basal flower stalk explants showed a higher NSHO than apical explants. DeMason (1979) showed for onion that the entire inflorescence is a single internode and grows by a basal intercalary meristem. The majority of the growth occurred in the lower third of the inflorescence. These basal meristematic cells were responsible for regeneration of shoots from basal explants of leek inflorescences. The meristematic activity is highest in the lower part of the flower stalk, indicating that shoot regeneration is highest from basal explants. Meristematic activity and subsequent PREX and NSHO declined with increased flower stalk length (age).

Histological observations revealed that the adventitious shoots arise from single cells. Because of the one cell origin of the regenerated flower stalk shoots, there is a risk for somaclonal variation. Until now, no morphological deviations were observed among leek plants obtained via the flower stalk method. The tetraploid nature of leek probably reacts as a buffer for induced variation.

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