Induction, maintenance and utilization of male sterility in leek (*Allium ampeloprasum* L.)

Inductie, handhaving en gebruik van mannelijke steriliteit in prei (*Allium ampeloprasum* L.)

Promotor:	dr. ir. E. Jacobsen hoogleraar in de plantenveredeling, in het bijzonder de genetische variatie en reproductie
Co-promotor:	dr. ir. A.M. van Harten universitair hoofddocent plantenveredeling

Ben Silvertand

Induction, maintenance and utilization of male sterility in leek (*Allium ampeloprasum* L.)

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STELLINGEN

1. *Allium ampeloprasum* L. is een betere wetenschappelijke naam voor prei dan *Allium porrum* L..
   Dit proefschrift

2. De opmerking van Pearson (1981) dat er meer aandacht besteed moet worden aan het opsporen van "natuurlijke" vormen van CMS geldt in het bijzonder voor prei.
   Pearson OH (1981) Hort Sci 16:482-487

3. Voor het kunstmatig induceren van CMS is in principe elke methode geschikt die voor recombinatie van het mtDNA zorg draagt.
   Dit proefschrift

4. Niet de inductie, maar de selectie van een CMS mutant is het belangrijkste obstakel in het verkrijgen van CMS.
   Dit proefschrift

5. De beste weg in prei van mannelijke fertiliteit naar mannelijke steriliteit is waarschijnlijk die van mannelijke steriliteit naar mannelijke fertiliteit.
   Dit proefschrift

6. CMS moet gezien worden als een stadium in de co-evolutie van kern en mitochondriën en niet als een lethale mutatie.
   Saumitou-Laprade en medewerkers (1994) Tree 9:431-435

7. Plantenveredeling is de wetenschap en de kunst van effectief management van genetische variatie en van een flinke dosis geluk.

8. De bewering van M.K. Ghandi dat de wereld voldoende te bieden heeft voor ieders behoeften, maar niet voor ieders hebzucht, is zonder meer nog steeds waar.

9. Onder het mom van een beter milieu kun je tegenwoordig alles verkopen.

10. De opstandige studenten van vandaag worden meestal de brave burgers van morgen.

11. In deze tijd is de beste promotie een nieuwe baan.

Stellingen behorende bij het proefschrift "Induction, maintenance and utilization of male sterility in leek (*Allium ampeloprasum* L.)" door Ben Silvertand, in het openbaar te verdedigen op vrijdag 12 april 1996, te Wageningen.

Voor Esther

Abstract

This thesis describes research work performed in leek in relation to efforts to obtain or exploit a male sterility system for hybrid leek breeding. Male sterile plants can be found in every leek cultivar. The male sterility trait can be explained by a nuclear monogenic inheritance or by a nucleo-cytoplasmic inheritance. The existence of a nucleo-cytoplasmic inheritance remains speculative unless the maintainer genotype will be identified. No source of CMS has been found, sofar, in leek or related forms of *A. ampeloprasum* L.

Mutation breeding experiments during an extended period of time, using chemicals or somaclonal variation, did not result in the induction of cytoplasmic male sterile mutants in leek.

The small chances for fixation of a mutation in a mitochondrial genome population and problems concerning the identification and recovery of the cytoplasmic mutation at the plant level may have been the major obstacles in obtaining a cytoplasmic male sterile mutant.

An efficient callus regeneration system and the selection of leek plants with improved regeneration ability are described in this thesis. The availability of a regeneration system is an essential step in the application of somatic hybridization, somaclonal variation and transformation techniques in leek.

Vegetative propagation of nuclear male sterile genotypes remains up till now the most realistic option for producing leek hybrids. A new *in vitro* method for mass clonal propagation of leek, based on flower stalk explants, was developed and is described in this thesis. This method offers a realistic opportunity to leek breeders for producing leek hybrids, until a less expensive genetic system for producing male sterile lines would become available.

Contents

CHAPTER 1

General introduction

Taxonomy

Leek (*Allium ampeloprasum* L.) is a member of the genus *Allium*, which probably consists of more than 600 species (Hanelt 1990, Jones 1991). There is considerable disagreement about the taxonomic position of the genus. Linnaeus already placed it in the family of the *Liliaceae* and Hutchinson (1959) and Traub (1957, 1968) in the *Amaryllidaceae*. A third alternative is to place the genus in a new family, *Alliaceae* (Agardh 1858; Dahlgren et al. 1985; Hanelt 1990). The subdivision of *Allium* into subgenera and sections, based on the morphology of the plant, placed leek in the subgenus (sect.) *Allium* (Von Bothmer 1974). A second matter of disagreement is the species name. Leek has been known since Linnaeus as *Allium porrum* L, but nowadays some taxonomists prefer a broader species concept and place leek in the large *Allium ampeloprasum* complex. Wild taxa from the Mediterranean region and southwest Asia as well as the cultivated forms, namely leek (*Allium porrum*), kurrat (*Allium kurrat*), pearl onion (*Allium porrum* var. *sectivum*), and great-headed garlic (*Allium ampeloprasum*), are considered components of this variable species (Van der Meer and Hanelt 1993). According to De Wilde-Duyfjes (1976) the *Allium ampeloprasum* complex further includes *A. ampeloprasum*, *A. bulbiferum*, *A. commutatum*, and *A. polyanthum*. Von Bothmer (1974) further includes *A. bourgeaui* and Davies (1992) *A. babingtonii*, *A. pardoi*, *A. pyrenaicum* and *A. scaberrimum*. In this thesis the name *A. ampeloprasum* L. will be adopted for leek, because it was found in this research that leek showed a good crossability with other relatives of the *ampeloprasum* complex and that fertile progenies could be obtained.

Leek is considered to have originated from the wild forms of *A. ampeloprasum* (Jones 1991). These wild forms differ from leek and kurrat by the production of a large bulb which consists of two cloves (Jones and Mann 1963). According to Vavilov (1926), the primary centre of origin of leek is the Near East and the Mediterranean region. Wild plants of this species occur in the Mediterranean area from Portugal and northwest Africa in the west to Turkey, Syria, northern Iraq, and western Iran in the east (Stearn 1978, De Wilde-Duyfjes 1976). According to Masefield et al. (1969) *A. ampeloprasum* is also a native of the Atlantic islands of the Azores, Canaries, Cape Verdes and Madeira and possibly native in a few areas on the southern coasts and off-shore islands of England and Wales.

Further taxonomic research and crossing experiments are required to gain further insight concerning the position of leek within the *Allium*

ampeloprasum complex and among its congeners within the section *Allium*.

Cytogenetics

The various members of the *Allium ampeloprasum* complex show different ploidy levels, i.e., $2n$ = 24, 32, 40, 48, or 56 (Van der Meer and Hanelt 1990, Jones 1990). However, tetraploids and hexaploids are predominant in wild populations (Von Bothmer 1974). The cultivated leek is a tetraploid ($2n$ =4x=32) (Levan 1940). There is some discussion whether the leek is an autotetraploid or an allotetraploid. Levan (1940), Kadry and Kamel (1955), Murin (1964) and Berninger and Buret (1965) share the opinion that leek is an autotetraploid. Koul and Gohil (1970) proposed that three parental genomes were involved with the formula AAA'A'', but their research was carried out with wild *A. ampeloprasum* plants. According to Stebbins (1947) natural autopolyploids are likely to be of segmental type, i.e. some parts of their genome behave as autopolyploids and other parts as diploids. Gohil (1984) suggested that tetrasomic inheritance occurs for most genes. This is confirmed by the formation of quadrivalents during the earlier stages of meiosis. It is well established that in case of tetrasomic inheritance the alleles have less chance of establishing a homozygous state. Leek almost shows exclusively bivalent formation at meiosis, apart from some quadrivalents in early stages of the meiosis, and thus there are no problems with chromosome segregation or seed set. The reason for the regularity of meiosis is thought to be the localisation of chiasma formation, proximally at sites adjacent to the centromere (Kollmann 1972). The position of these chiasmata is the reason for their inability to disturb the gene combinations in the two arms of each chromosome. As such, all the genes present on the chromosome arms distal to the region where the chiasmata are formed, remain together and may start behaving as supergenes whose strong linkage can not be broken, and as a consequence only a negligible amount of recombination takes place (Gohil 1984). According to Potz (1987) the chiasma localisation plays a major role in the diploidisation of the autotetraploid leek. Consequently an undisturbed meiosis resulting in a good fertility and seed set is found. In spite of this limited recombination, one of the most striking features of leek is its strong sensitivity to inbreeding depression (Schweisguth 1970; Berninger and Buret 1967).

Another peculiarity of leek cytology was reported by Vosa (1966) and concerned the relationship between the presence of B chromosomes and the seed germination. These B chromosomes are found in frequencies up to 30%

in some cultivars, and were shown to give a slight enhancement of seed germination (Gray and Thomas 1985).

Cultivation of leek

Leek is grown practically in all soil types, the most important requirement being a loose texture. On peat soils, yields are usually high but quality is bad. Traditionally, sandy clay soils are the most suitable for leek cultivation in the Netherlands, because harvesting is difficult on more heavy soils in autumn and winter (Van der Meer and Hanelt 1993).

The area of leek in the Netherlands has remained constant during the 1980's, whereas from 1990 onwards an increase in the crop area can be observed (Table 1). Farmers switched over from arable crops to market garden crops, such as leek, as a result of overall overproduction and low prices (De Kraker 1993). Furthermore, the increasing mechanical harvest of leek made it economically necessary to further increase the production area. The bulk of the leek production (± 90%) in the Netherlands is concentrated in the southern provinces of Noord-Brabant and Limburg.

The large number of different leek cultivars makes it possible to sow the crop from December to June and to harvest from June to May in the next year. The cultivation of leek is divided, according to the time of harvest, into three periods, i.e., summer-, autumn- and winter period. A further subdivision is made into very early, early and normal summer cultivation, early and late autumn cultivation and normal winter and late winter cultivation (Anonymous 1994). Sowing of summer leek varieties starts in December and continues until March. The autumn varieties are sown in March and the winter varieties from April to the middle of May. The summer varieties are raised in a heated greenhouse, whereas the autumn and winter varieties are sown under 'cold glass' or in an outdoor bed. At the end of the raising period leek plants must have the thickness of a pencil (5-6 mm). In the Netherlands and other European countries, leek plants are transplanted after a raising period of about 12 weeks to the production fields. This transplanting method gives growers the opportunity to reject weak-growing plants. In the United Kingdom direct-drilling, without transplanting, is more frequently applied. Automatic planting machines and machines for punching planting holes in combination with manual planting are available. Harvest can be performed manually or by a share lifter. When harvested by hand, the plants are usually lifted mechanically; consequently the roots are cut, the outer damaged and senescent leaves are removed, the remaining leaves shortened, and the plants

are packed into boxes. When mechanical harvesting is used, hand cleaning and machine washing are necessary (Van der Meer and Hanelt 1990). The 'Śummer' leek cultivars can be harvested from early June to September. From September till December the 'Autumn' leek takes over, followed by the 'Winter' leek, which can be harvested from January to April. In order to fill up the gap in the sales market during May and June, leek plants are stored in cold rooms or freshly harvested leek plants are imported from southern European countries. In Belgium a special method of cultivation, called 'Oude Jonkman' or 'Stekprei' is sometimes applied to fill up the gap in the production in May and June (Van Parys 1991). This cultivation begins by sowing in July and August and transplanting in October and November and subsequently harvesting in May and June. The quality, growth and yield of the harvested plants strongly depend on weather conditions.

Harvested leek plants are mostly sold at vegetables auctions in the Netherlands, and subsequently find their way to the consumer via market places and supermarkets. About 10 percent of the available leek production is processed by the industry. The total exported quantity of leek and the percentage of the exported leek on the total leek production are increasing year after year (Table 1). The most important export market for Dutch leek is Germany which accounted for about 51% of the leek export in 1993. Other important Dutch foreign trade markets for leek are: France (14%), Sweden (10%), U.K. (5%), Belgium, Luxembourg (5%) and Italy (3%).

France is the country with the largest area and highest leek production within the European Union (E.U.), followed respectively by Belgium, the Netherlands, U.K. and Spain (Table 2). Germany, Greece, Italy and Denmark show a relatively small area of leek production. An increase during the last decade in area and production of leek was observed mainly in Belgium and the Netherlands, whereas other countries of the E.U. showed a constant area and production.

Table 1. Production of leek in the Netherlands from 1975 to 1994.

	Marketing figures (x 1000 kg)					
Year	Area (ha)	Production	Import	Export	Fresh cons.	Industrial proc.
1975	1 283	47 602	830	7 123	33 920	6 806
1980	1 645	51 039	2 536	7 220	38 989	6 753
1985	2 856	57 602	4 025	12 117	43 772	5 464
1986	2 785	69 733	3 643	15 468	48 929	8 234
1987	2 832	63 828	3 904	15 817	44 801	6 805
1988	3 025	79 713	2 227	23 919	46 476	10 358
1989	3 007	82 235	2 342	29 735	45 592	8 710
1990	2 896	94 312	2 353	40 091	47 333	8 901
1991	3 799	98 793	4 410	45 099	47 926	10 035
1992	4 683	114 299	3 101	55 907	48 638	11 085
1993	4 495	109 200	4 918	52 544	43 881	-[2]
1994	4 249	64 080[1]	-	-	-	-

[1] production estimation until September
[2] not known
(Figures derived from Anonymous (1991-1994a), Anonymous 1990-1994, Anonymous 1993 -1994)

Table 2a. Area of leek within countries of the European Union (1980-1992).

	Area of leek (x 1000 ha)											
Year	FRA[1]	BEL	NLD	UKD	ESP	BRD	GRE	ITA	DMK	LUX	POR	TOTAL
1980	9.9	2.3	1.6	1.7	-[2]	1.5	1.8	1.2	0.5	0.0	-	20.9
1985	9.7	3.1	2.9	2.6	2.9	1.5	1.8	1.3	0.4	0.0	-	26.2
1986	9.8	3.3	2.8	3.1	2.8	1.7	1.7	1.2	0.4	0.0	-	27.0
1987	9.8	3.3	2.8	3.1	3.0	1.7	1.7	1.3	0.4	0.0	-	27.1
1988	9.8	2.9	3.0	2.9	3.1	1.7	1.6	1.3	-	0.0	-	26.3
1989	8.8	3.8	3.0	3.1	3.4	1.6	1.7	1.4	-	0.0	-	26.9
1990	8.6	4.3	2.9	3.4	3.0	1.7	-	1.3	-	0.0	-	25.2
1991	9.0	4.8	3.8	3.3	-	1.5	-	-	1.3	0.0	-	23.7
1992	-	-	4.7	3.2	-	1.9	-	1.2	-	-	-	-

[1] FRA = France, BEL = Belgium, NLD = The Netherlands, UKD = United Kingdom, ESP = Spain, GRE = Greece, ITA = Italy, DMK = Denmark, LUX = Luxembourg, POR = Portugal
[2] not known

Table 2b. Production of leek within countries of the European Union (1980-1992).

	Production of leek (x 10^6 kg)										
Year	FRA[1]	BEL	NLD	UKD	ESP	BRD	GRE	ITA	DMK	LUX	POR	TOTAL
1980	217	69	53	36	-[2]	33	41	32	8	-	-	489
1985	229	87	66	60	56	39	36	30	7	-	-	610
1986	225	107	67	71	55	43	37	34	7	-	-	647
1987	226	102	76	72	62	43	43	37	9	-	-	669
1988	213	102	82	71	68	43	39	35	-	-	-	653
1989	198	155	92	74	72	48	39	38	-	-	-	715
1990	196	146	89	70	65	46	37	36	-	-	-	685
1991	204	137	99	72	-	48	-	35	-	-	-	595
1992	213	164	114	78	67	55	39	33	-	0	-	763

[1] FRA = France, BEL = Belgium, NLD = The Netherlands, UKD = United Kingdom, ESP = Spain, GRE = Greece, ITA = Italy, DMK = Denmark, LUX = Luxembourg, POR = Portugal

[2] not known

(Figures derived from: Anonymous 1991-1994b)

Breeding

Early History

Leek (*Allium ampeloprasum* L.) is a vegetable that has been cultivated from the earliest times. It is not naturally found in the wild, but it is considered to have originated from wild forms of *A. ampeloprasum*, a species which originates from the Near East (Jones and Mann 1963). This region was one of the cradles for the evolution of agriculture. Domestication of plants started in this area about 12000 years ago (Harlan 1992). It is obvious that long before this time, hunting and gathering man must have collected the wild forms of leek for dietary and medicinal purposes.

The garden leek was a popular vegetable in the ancient Near East (McCollum 1976; Zohary and Hopf 1993). References to leek as food, medicine or religious objects can be traced back to the first Egyptian Dynasty (3200 B.C.). Herodotus travelled through Egypt some time around 450 B.C. and he listed the amounts of leeks provided for the labourers building the pyramid of Khufu (Cheops) around 2500 B.C. (Davies 1992). According to Täckholm and Drar (1954) in the Sumerian period, around 2000 B.C., a distinction was made, already, between kurrat and leek. The leaves were the edible part of kurrat, whereas the pseudostem of leek was consumed. Ar-

chaeological findings indicate that ancient leek was an intermediate between our present leek and wild *A. ampeloprasum* (Zohary and Hopf 1993). Ages of domestication that followed, resulted in the development of a plant with distinct features. Outside Egypt few remains are left, but include either wild or cultivated material from Early and Middle Bronze Age Jericho (Hopf 1983). A passage from the bible (Number 11:5) contains a complaint by the children of Israel stranded in the wilderness after their exodus from Egypt (1500 B.C.): 'We remember the fish we did eat in Egypt freely, the cucumbers and the melons and the leeks and the onions and the garlic.'

A further link with the past comes from the Greek word for leek: *prason*, from which we derive *ampeloprasum*, *ampelo* meaning vine; i.e. the *Allium* that grows in vineyards. The Greek herbal of Dioscorides distinguished between *prason* (leek) and the wild ancestor *ampeloprason*.

Fig. 1. Roman vegetable stall. Vegetables that can be identified are cabbage, kale, garlic, leek and onions. Derived from White (1970).

The illustration (±512) of leek in this herbal shows a large bulb at the basis of a large pseudostem (Gunther 1968). After the Greek era, the Romans took over the leading role on the stages where world's history is performed.
Their Latin word for leek was *porrum*. The Romans split *porrum* into two forms: *porrum capitatum* and *porrum sectilis*. From the form *porrum sectilis* the leaves were cut and consumed and this type must have referred to kurrat. This classification was maintained during the Middle Ages and is used by the many herbalists, who confused *porrum sectilis* with the form we call chives (*Allium schoenoprasum* L.). McCollum (1976) also refers to chives in this respect. The Roman history writer Pliny the Elder states that the best leek came from Egypt, and in the second place from Orthe, a town of Asia minor, now called Guzelhizar. Aracia in Italy, now called Riccia, was also known for leek in Pliny's time (Phillips 1822; Gibault 1912). The emperor Nero had great pleasure in leek, hoping to improve his singing voice, and therefore he was called in scorn *Porrophagus* (Gerarde 1597). Apicius (±230) considered leek and garlic to be more important in Italy than onion. A sculpture of a Roman vegetable market-stable shows a bundle of leek plants (Fig. 1). For centuries, Rome was the centre of the civilized world and the Romans must have brought the leek, together with civilization, to the edges of their empire.

Medieval Europe was well acquainted with this vegetable. Onions, garlic and leek, all common vegetables were mentioned by Chaucer (±1340) (McCollum 1976). Leek was grown and harvested in the Italian Po-area, as can be observed from a picture in the herbal 'Tacuinum Sansitatis' of Botlân (1380-1400) (Anonymous 1981) (Fig. 2). The herbal 'Hortus Sanitatis' of Giovanni da Cuba (1484) provides us with a picture of the cultivation of leek in Italy (Fig. 3) (Anonymous 1980). It is remarkable to see that the leek plants were planted in a very wide spacing. The leek on both figures have a similar appearance; their shafts (pseudostem formed by the leaf sheaths enclosing the young folded leaf blades) are long and bulbous at the bases. Crescentius (1493, 1518) gave a detailed description of the cultivation of leek with a relatively short shaft, and shows that the roots were cut before transplanting, and that the plants were transplanted into trenches for obtaining blanched stems (Fig. 4). The sizes of the plants indicate that transplanting occurred after a long raising period, as compared to the present 12 weeks raising period. A spread in the harvest was achieved by different sowing times. From a study of pictures in medieval herbals it can be concluded that the leek in this period was still very bulbous and bore

Fig. 2. Harvest and preparation of bundles of leek plants in the Italian Po-area. Illustration derived from the herbal 'Tacuinum Sansitatis' of Botlân (1380-1400).

either a long or short shaft. Gerard (1597) reported that leek was common everywhere in England as well as in other European countries. Noteworthy in this respect is the national interest of the Welsh in leek, as it goes far back to the sixth century.

The Welsh still continue to wear leek on St. David's day in commemoration of a victory which they obtained over the Saxons in 640 A.D., and which they attribute to the leek they wore to distinguish themselves in the battle (Phillips 1822; Masefield et al. 1969). The herbalists Braunschweig (1533), Fuchs (1549), Dodonaeus (1563), Gerard (1597), Tabernaemontanus (1613) and Munting (1696) distinguished two leek types: *Porrum capitatum* (leek) and *Porrum sectilis* (chives). Some special virtues were ascribed by them to the crop: it brings up humour that lies in the chest, it cleans the lung pipes, it cures bitings of insects, it removes noise in the ears, it causes "the natural disease of the woman" and prevents pregnancy. However they also warned

Fig. 3. Cultivation of leek in Italy. Illustration derived from the herbal 'Hortus Sanitatis' of Giovanni da Cuba (1484).

for its dangers: it heats the body, it causes farts, it works laxative, it causes troublesome and terrible dreams, it offends and darkens the eyes, it gives a silly and dark face and it causes abortions. In the New World, Cortés noted leek growing, and by 1775 the Choctaw Indians were cultivating it (Davies 1992).

Early landraces and varieties

The early leek varieties, which, in fact were actually landraces, were highly variable in appearance and had different local names. These early leek landraces were adapted to local conditions, and propagated by mass selection. Leek growers harvested their own seeds from selected plants and individual leek plants were selected on the basis of high yields (thick and large pseudostems or shafts). Furthermore, because of the biennial nature of the plant, natural selection for winter-hardiness must have occurred in an early time period in places with severe winter conditions. Winter hardiness demands a slow growth and plants with a short (below the surface of the soil) pseudostem and broad leaves have a natural advantage, because those plants are better adapted to extreme cold conditions.

Fig. 4. Transplanting of leek plants in trenches in Germany. Illustration derived from Crescentius (1493).

Therefore, it seems obvious that, for instance, in northern European countries selection occurred for late and winter hardy varieties with a short pseudostem, while the selection of early and fast growing landraces with a long pseudostem was restricted to southern European countries with less severe winter temperatures. Apart from the distinction between *porrum capitatum* and *porrum sectilis* by Greek and Roman writers, no written evidence of different leek landraces can be found for a very long period of time. It was De Combes (1752) who, for the first time, mentioned two leek landraces in France: Le Long and Le Court. Both types also could be found in medieval herbals from an earlier period and on paintings from the 17^{e} century (Fig. 5).

Fig. 5. Painting from Speyer (1681-1752) showing some vegetables and fruits, including leek with a short pseudostem.

It seems that Le Long was most often cultured because of its long shafts and subsequently high yields. Le Court exhibited a strong winter hardiness and was recommended for a late harvest. According to Helm (1956) Le Long referred to a leek with a very long shaft derived from Syria and Palestine, while Le Court originated from Iran. It remains doubtful whether this information is correct. There is also the possibility that natural selection for winter hardiness in northern European countries caused the development of a type of leek able to resist severe winter conditions. A leek with a slow growth, dark foliage, broad leaves, and relatively short and thick shafts had, in this respect, the best chances of surviving severe winter circumstances. A very famous landrace was the Musselburgh or Scotch Flag, originating from Edinburgh in Scotland and known since early 19th century (Loudon 1823). Probably the Musselburgh was a selection from the London Flag. This Musselburgh showed a wide variation in the length of the pseudostem. Loudon (loc. cit.) also mentioned the Londoner leek with broad leaves. Vilmorin-Andrieux (1856) distinguished between several leek varieties and also mentioned a long shafted winter leek. It is difficult to understand, in this respect, why the long shafted variety showed a better winter hardiness than the short one. This leek was known in France, the U.K. and Germany as

resp. Poireau long (d'hiver de Paris), Very long winter leek or Sehr langer Winter-Porree. According to Gibault (1912) this variety was a selection from Le Long. Le Court was also known as Gros du midi, Broad Flag, London Flag or American Flag and in a later period referred to as French summer (Loudon 1823; Vilmorin-Andrieux 1856, 1925; Becker-Dillingen 1938). The famous Dutch Brabander Winter or the Flanders winter leek from Flanders were winter-hardy varieties (Vilmorin-Andrieux 1856; 1925). Another winter-hardy selection, cultured since 1874, was derived from the famous French Gros court de Rouen and known in France, Belgium, England and the Netherlands as the king of the leek; The Carentan or The Monstrous Carentan (Gibault 1912; Bois 1927). This variety decorated the Dutch variety lists until 1951, and was recommended in the Netherlands as an autumn type and in France as a winter type. Both the Musselburgh and the Carentan were also popular varieties in the USA at the beginning of this century (Cox et al. 1927). Benary (1911) in Germany distinguished between summer and winter leek types and recommended the French summer and Bulgarian giants for cultivation during the summer and autumn period, while the Brabander and the Carentan were suitable winter varieties in this time. Shortly, before the second World War the popular autumn variety Elephant was introduced. This variety was derived from the Belgian winter variety de Liège (Van Doormalen, Nunhems Zaden, pers. comm.). Elephant gave high yields but, unfortunately, was bulbous at the basis. Figure 6 demonstrates the variability in lengths of shafts, erectness of the leaves and bulbousness between summer, autumn and winter varieties at the beginning of this century. At the second half of the 20^{e} century numerous new selections originated in the Netherlands, France, Belgium, U.K. and Germany after mass selection in existing varieties. The large genetic variation within these varieties made mass selection an attractive approach for further improvement of the leek and the selection pressure forced the varieties to higher standards, but insurmountable, at the same time, reducing the genetic variation within these selections. Selection for winter-hardiness, long shafts in winter varieties, erectness of the leaves and dark leaf colours became desirable traits. Improvement of the winter hardiness was achieved by the introduction and selection within Belgian varieties (i.e. de Liège and Mechelse Winter). From the 1960's onwards family selection replaced mass selection and Alaska became the first "modern" leek variety (Barends, Royal Sluis, pers. comm.), soon followed by many other modern leek varieties. Leek breeders

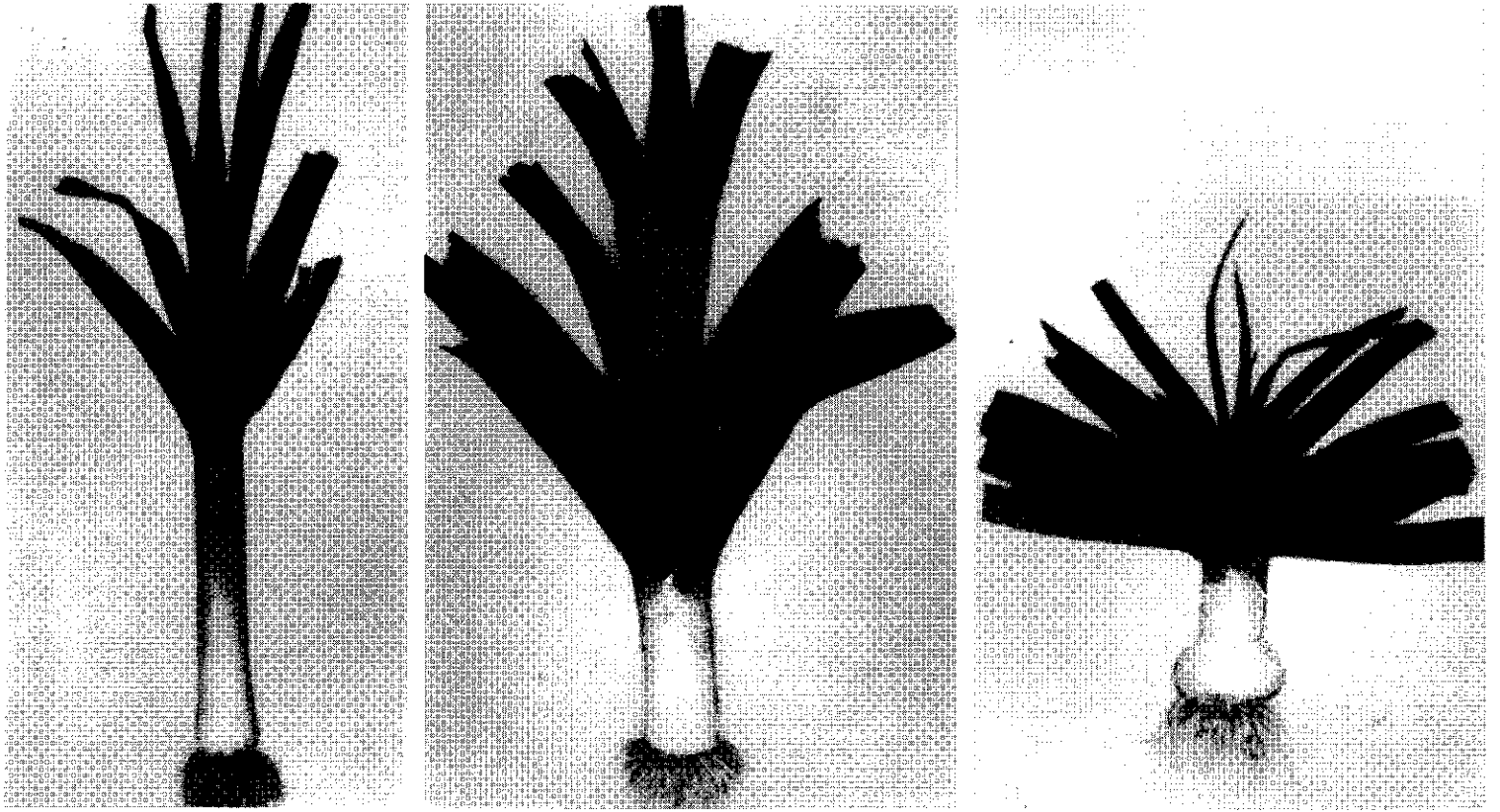

Fig. 6. Variability in shaft lengths, erectness and bulbousness between summer, autumn and winter varieties of leek during the first part of the 20^{e} century; French Summer (left), Elephant (middle), Brabander (right).

subsequently started to perform crosses between the different types of leek varieties (i.e. summer, autumn and winter), and, as a result, it became difficult to distinguish between the different types.

As a consequence of the intensive selection within the former landraces a severe genetic erosion has occurred and many local strains, especially in Belgium and the U.K., have been replaced by modern varieties. According to Van der Meer and Hanelt (1990) the loss of genetic diversity, inevitably, will hamper breeding for resistance to pests and diseases. However, thanks to the conservation work of gene banks, genetic resources may be saved. At the very moment the genetic improvement of leek by mass and family selection is reaching its breeding limits, and the time is ripe for hybrid leek breeding in order to deal with the present market demands. Before the turn of this century, after a period of 5000 years of leek cultivation and genetic modification, the first hybrid leek varieties are to be expected and, probably, will replace all existing leek varieties, but they will never reach the fame and glory of the old Musselburgh or Carentan, or for the "younger" people, among us, the Elephant or Goliath.

Breeding objectives and prospectives

At present, the leek cultivars are classified, according to their harvest period, as summer, autumn and winter types. These cultivars show differences in length of the shaft, leaf colour, earliness and winter hardiness. For summer varieties earliness and bolting resistance are the most important characteristics. Breeding emphasis for the autumn and winter cultivars is focusing on increased shaft length, increased erectness of the leaves, absence of bulbing of the shaft, a narrow shaft base, improvement of winter hardiness and a dark green leaf colour. The dark green leaf colour is also becoming an attractive fashion colour in early (autumn and summer) varieties. This dark colour, however, is not only a fashion colour but also positively correlated with disease resistance and prevents deterioration of the plant under bad weather conditions, especially during autumn and winter.

General breeding aims are: improvement of uniformity, higher yields and resistance against pests and diseases. In particular, lack in uniformity is a major limiting factor in the marketability of the crop (Pink 1993). Leek is attacked by many pests and diseases. Onion fly (*Delia antiqua*), thrips (*Thrips tabaci*), leek moth (*Acrolepiopsis assectella*) are the most important pests, whereas, white tip (*Phytophthora porri*), purple blotch (*Alternaria porri*), leaf blotch (*Cladosporium allii-porri*), black stripe (*Leptotrochila*

porri) and rust (*Puccinia allii*) are the diseases most frequently observed in leek. The attacks from *phytophthora*, *puccinia* and *thrips* increase year after year, and resistant cultivars are desirable (Wietsma and De Vries 1990). Year round growing of the crop provides a 'green bridge' between crops for pests and diseases and will facilitate their expansion (Pink 1993).

The importance of leek in several European countries is increasing annually. However, the crop shows, as has been mentioned before, some major imperfections, e.g. lack in uniformity and severe disease problems. The available breeding systems (mass and family selection) are unsuitable to solve these problems. The most suitable breeding system able to cope with these problems seems to be hybrid leek breeding. Hybrid leek breeding is hampered, however, by the lack of an economically suitable system of producing male sterile lines for hybrid seed production. Male sterile lines are desirable in order to prevent selfing in the seed producing lines. A system based on cytoplasmic male sterility might prove to be the best choice for the genetic improvement of leek in the future.

Breeding experiments

Berninger and Buret (1967) reported that leek is an outbreeding species with up to 20% of self-fertilization. Schweisguth (1970) made inbred lines and observed a severe loss of vigour and seed production after selfing. Many deleterious characters were observed after selfing. A striking feature of the inbred lines was their improved uniformity. Male sterile plants were discovered in selfings and in open-pollinated varieties. Experimental hybrids between I_1 plants showed a good vigour. In later leek breeding experiments, Schweisguth (1973) investigated the heritability of the shaft length, leaf length and leaf width and found a positive genetic correlation between shaft length and leaf length and high heritabilities for shaft length and leaf length. For breeding Schweisguth (loc.cit.) advised to select the length of the shaft and the length of the leaf on both parents. A general conclusion was that hybrids between clones were the best varietal structure for leek, but that propagation of male sterile lines caused problems (Schweisguth 1983).

Gagnebin and Bonnet (1979), who also noticed a strong inbreeding depression after selfing as well as a strong heterosis effect in their hybrids, obtained cold resistant material with long shafts, showing that the linkage between cold resistance and short shafts could be broken.

Kampe (1980) made experimental hybrids and obtained both increased yields (up to 78%) and shaft diameters (up to 43%), as compared to the

original cultivar. Heterosis for shaft length was not found in his experiments. In order to facilitate the production of hybrids, he recommended searching for male sterile plants in extensive seed production fields. He further suggested that those plants could then be multiplied by bulbils or by meristem culture. Baumunk-Wende (1985-1990) obtained high percentages of male sterile plants in inbred lines of commercial varieties. The establishment of hybrids was hampered by the lack of a suitable *in vitro* system for a large scale multiplication.

Van der Meer and Van Bennekom (1973, 1976) and Van der Meer (1982) experimented with the gametocides GA_3 and $GA_{4/7}$ in order to create male sterile plants of onion and leek. Male sterility in both crops was induced; however a severe reduction in seed production was an undesirable but common side-effect. Resistance against the leek yellow stripe virus (LYSV) was transferred from kurrat to leek by common crossing experiments (Van der Meer and Van Bennekom 1980, 1981; Van der Meer 1980).

Gray and Steckel (1986) compared self-pollination with open-pollination in leek and observed that open-pollination gave higher mean seed weights and lower seedling losses during plant-establishment than self-pollination. Smith and Crowther also reported a severe inbreeding depression after selfing, but they also showed that obtaining relatively vigorous inbreds was feasible. Their hybrids outperformed the open-pollinated cultivars considerably, especially for yields. They concluded that the exclusion of inbreeding, made possible by the utilisation of male sterile lines, was the main reason for the yield improvement. For practical breeding it was suggested that inbreeding should not go beyond the I_1 level when developing pollinator lines.

Outline of the thesis

The utilization of male sterility is the key to hybrid leek breeding. Therefore, the establishment, maintenance and application of this trait in leek are the main objectives of the present research. **Chapter 2** supplies the reader with basic information about male sterility and provides the foundation for the following chapters. This information is supplemented and compared with results of some of our preliminary experiments. Furthermore, in this chapter, crossing experiments within the *Allium ampeloprasum* complex are described.

The best genetic system for the development of hybrid cultivars in leek is a system based on cytoplasmic male sterility (CMS). An extensive body of evidence in literature indicates that CMS is encoded by the mitochondrial

(mt) genome and that recombination in this mt-genome is an important factor for the establishment of this trait. In order to promote the occurrence of this CMS trait, it seems justified to apply mutation methods which are known to enhance recombination processes in the mt-DNA. In **chapter 3**, a central subject of this thesis, attention is focused on the induction of CMS by chemical mutagens.

Research work in leek, especially when flowering plants are involved, is delayed by the fact that leek plants normally flower in the second year of their development. In order to complete this research within the limited period available, it was necessary to develop a one-year cycle for leek. The struggle, against nature's impulses to prevent this speeding up of the developmental processes in leek, is presented in **chapter 4**.

An alternative approach for the establishment of leek hybrids is the *in vitro* propagation of male sterile plants via flower stalk segments. A successful and powerful *in vitro* multiplication method is presented in **chapter 5**.

The lack of an efficient regeneration system for leek was an obstacle for the progress in it's genetic modification. An efficient callus regeneration system and selection of good regenerating genotypes are required for the successful application of somatic hybridization and transformation techniques in leek. The development of a system of organogenesis and somatic embryogenesis was a primary and necessary step in the genetic reconstruction of leek and is described in **chapter 6**. Much variation in callus formation and plant regeneration occurred between different leek cultivars and among the genotypes of a cultivar in the tissue culture experiments. In the framework of the research work and in order to study practical applications, the mode of the genetic control of callus formation and plant regeneration in leek was evaluated and genotypes with improved *in vitro* shoot regeneration were established. These results are summarized in **chapter 7**.

Chapter 8, the general discussion, encounters the possibilities and restrictions when utilizing male sterility and highlights new approaches, on behalf of hybrid leek breeding, for future research.

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CHAPTER 2

Male sterility in leek (*Allium ampeloprasum* L.)

Male sterility

Male sterility (MS) in plants is generally described as the inability of a plant to produce or release functional pollen (Dorsey 1914). This broad term covers all cases of infertility that results from irregularities in the male sexual reproductive system. The MS trait is used by plant breeders to control pollination, in order to exclude selfing and to circumvent hand emasculation in the line (female) that produces the hybrid seed. Classification of MS can be made on a phenotypic and a genotypic basis (Kaul 1988).

The phenotypic anomalies are categorised into three types: (1) 'structural male sterility'; characterized by structural anomalies which predominantly occur in the male sex organs, resulting in the absence, mis- or mal-formation of the microsporogenous tissue; (2) 'sporogenous male sterility'; characterized by mis- or mal-formed sporogenous tissue and resulting in non-, mis- or malformation of pollen; and (3) 'functional male sterility'; characterized by the formation of functional pollen incapable of fertilization due to some external barriers (e.g. anther anomalies). MS plants also may show a combination of these phenotypic anomalies. For instance, in some barley, maize and tomato mutants, stamens are either reduced or deformed. In them, a few pollen mother cells develop in which meiotic breakdown may occur either at the beginning or at the end of microsporogenesis. Thus they share features of structural and sporogenous types. Of the three types mentioned, sporogenous male sterility is by far the most common and the only one that has played a major role in plant breeding and hybrid seed production (Kaul 1988; Lasa and Bosemark 1993).

On a genetic basis two basic types can be distinguished: (1) 'nuclear male sterility' also referred to as 'genic' or Mendelian male sterility (NMS), in which the male sterility is controlled by nuclear genes, and (2) 'cytoplasmic male sterility' (CMS), in which the MS is a result of a malfunctioning interaction between nuclear and organellar genes. According to Frankel (1973) and Kaul (1988) MS can also be determined by cytoplasmic factors only.

Male sterility in plants can also be the result of environmental conditions or chemical agents.

Nuclear male sterility

Nuclear male sterility (NMS) widely occurs in flowering plants and the majority of the male steriles have arisen as spontaneous mutants or have been

induced by mutagen treatments (Kaul 1988). Mutation from male fertility to male sterility is a frequently occurring phenomenon in many plant species. Nuclear male sterility has been found to be controlled by one or more genes and conditioned by monogenic recessive genes in the majority of the spontaneously arisen mutants (Kaul 1988; Lasa and Bosemark 1993). Kaul (1988) argued that male sterility in monocots is almost exclusively conditioned by recessive mutations. NMS provides a genetic means of emasculation, but an exclusively female line can not be obtained directly with this system. When the male sterility is controlled by a single dominant gene, only 50% of the progeny of a cross will be male sterile. If it is inherited in a monogenic recessive condition, as is most common, 50% male steriles are obtained when a male sterile is crossed to a heterozygous fertile plant. This is a major drawback, as 50% of the plants have to be removed during flowering. An earlier removal is in general not possible, because male fertile and male sterile plants can not be distinguished before flowering.

According to Rao et al. (1989) several approaches can be followed to overcome this difficulty: (1) a genetic method can be used for producing a marker phenotype that helps in the recognition of the male sterile plants (e.g. pleiotropy or easily recognizable linked marker genes, (2) cytogenetic methods; especially by employing special chromosome compositions (e.g. Balanced Tertiary Trisomic (BTT) lines in barley), (3) temporary restoration of male fertility by gibberellic acid and silver nitrate in order to achieve self pollination, and (4) manual pollination where sterility is due to indehiscent anthers. Dunemann and Grunewaldt (1991) described a method to utilize a monogenic dominant nuclear male sterility in broccoli (*Brassica oleracea* var. *italica* Plenck) by producing haploids and subsequent chromosome doubling to obtain homozygous male sterile plants, which give 100% male sterile progenies. NMS can also be artificially induced by transformation with an engineered chimaeric gene consisting of an anther-specific promoter associated with a male sterility gene. Mariani et al. (1990) constructed chimaeric ribonuclease genes that are expressed in the anthers of plants of tobacco and oilseed rape after transformation and which selectively destroy the tapetal cell layer, leading to male sterility. Different transformation experiments to create a NMS trait are reviewed by Vedel et al. (1994). A close linkage of the ribonuclease genes with a herbicide resistance gene makes early recognition feasible. Phatak et al. (1991) linked the functional male sterility gene in eggplant to the purple fruit colour and Tanksley et al. (1984) used enzyme marker loci linked with a nuclear male sterility locus.

Bassett (1991) found a linkage between the spindly branch character and male sterility in the common bean (*Phaseolus vulgaris* L.).

Cytoplasmic male sterility

CMS is a maternally inherited trait resulting in pollen abortion, while maintaining female fertility. With CMS no male fertile plants, as with NMS, have to be removed before flowering in a hybrid seed production field. Therefore, CMS is a valuable tool for plant breeders in the production of hybrid seed. CMS can arise through inter- or intraspecific crosses, protoplast fusions, spontaneously, after mutagenic treatment and by passage through tissue culture (Williams and Levings III 1992).

Based on its origin, CMS may be divided into autoplasmic CMS and alloplasmic CMS. Autoplasmic CMS refers to those cases where CMS has arisen within a species as a result of mutations in the cytoplasm, most likely in the mitochondrial genome. Alloplasmic CMS represents the cases where CMS has arisen from intergeneric, interspecific or intraspecific crosses and where the male sterility can be interpreted as being due to incompatibility or poor co-operation between the nuclear genome of one species and the organellar genome of another. This category also includes CMS in products of protoplast fusion (Lasa and Bosemark 1994).

CMS has been observed in more than 150 plant species from 50 genera and 20 families (Hanson and Conde 1985; Kaul 1988; Srivastava and Gupta 1991). Among these CMS types, more than one-half arose naturally, about 20% were discovered in intraspecific crosses, and the remainder in interspecific crosses (Frank 1989; Levings III 1990). Kaul (1988) concluded that only a very limited number of CMS types is established by means of mutation induction experiments and lists nine crop species in which CMS has been obtained by applying different chemical mutagens, X-rays and gamma-irradiation (see chapter 3).

There is a considerable body of evidence indicating that genetic determinants controlling CMS are confined to the mitochondrial genome and that nuclear genes control the expression of the CMS phenotype (Newton 1988; Hanson 1991; Srivastava and Gupta 1991). The mitochondrial (mt-) DNA of higher plants is more variable in structure and organization than the DNA of chloroplasts. MtDNA of higher plants is thought to consist of a master, circular chromosome, and subgenomic circular molecules (sublimons), generated by recombination between repeated elements in the genome (Atlan and Couvet 1993). However, there is still no definitive physical evidence that

a circular master molecule exists (Mackenzie et al. 1994). Small et al. (1987) suggested that sublimons are expected to show rapid molecular evolution, because mutational events are more quickly fixed by chance in a small population of molecules. Occasional amplification of sublimons could cause sudden genomic reorganization, possibly leading to the evolution of different cytoplasmic types, including CMS (Small et al. 1987; Newton 1988; Levings and Brown 1989). The mtDNA shows a remarkable high rate of rearrangements, predominantly inversions, but remains invariant in primary sequence (Palmer and Herbon 1988). The ability of mitochondria to fuse establishes a mitochondrial DNA population within a cell which is in recombinational equilibrium (Lonsdale et al. 1988). Mitochondrial genomes often contain chloroplast DNA sequences (Stern and Palmer 1984; Lonsdale 1987; Brears and Lonsdale 1988; Moon et al. 1988; Srivastava and Gupta 1991) and/or small circular and linear DNAs as well as single and double-stranded RNA episomes, apparently without any essential mitochondrial function (Williams and Levings III 1992).

In plants differences exist between the mitochondrial genome organization of male fertile and cytoplasmic male sterile lines. Many of these differences have no phenotypic effects. The CMS phenotype originates from changes in the mtDNA of the male fertile progenitors as a result of aberrant intra- or intermolecular recombination events and also by duplications and/or deletions, which have either modified existing genes or created new genes (Palmer 1985; Dewey et al. 1986; Sederoff 1987; Leaver et al. 1988; Makaroff and Palmer 1988; Pring et al 1988; Duchenne et al. 1989; Fragoso et al. 1989; Leaver 1989; Levings III and Brown 1989; Makaroff et al. 1989; Pingitore et al. 1989; Small et al. 1989; Dudareva et al. 1991; Köhler et al. 1991; Rouwendal et al. 1987; 1992; Spassova et al. 1992; Potz and Tatlioglu 1993; Vedel et al. 1994). In some species a mtDNA region (gene) and/or gene product associated with the sterile phenotype was identified (Hanson et al. 1988; Johns et al. 1992; Handa 1993; Vedel et al. 1994). The *T-urf*13 mitochondrial gene in the Texas cytoplasm of maize, encoding a 13 kDa polypeptide *URF*13, which is responsible for CMS and *Helminthosporium maydis* susceptibility, is one of the best characterized mitochondrial genome rearrangements, and is the result of both intra- and intermolecular recombinations and one or more co-transcribed mitochondrial genes with unmodified coding regions (Forde et al. 1978; Forde and Leaver 1980; Rottmann et al. 1987; Levings III and Dewey 1988; Levings III 1990; Williams and Levings III 1992). In petunia, CMS is correlated with the

presence of a 25 KDa protein designated *PCF*, which is encoded by the *pcf* gene (Nivison and Hanson 1989). Based on *T-urf* 13 and *pcf*, it can be envisaged that the CMS trait in other species might also be associated with single unusual chimeric genes. However it is interesting to note that there are plant species in which the CMS phenotype is associated with alterations in the primary structure of a number of mt genes, for example CMS-C in maize (Dewey et al. 1991), CMS in carrot (Scheike et al. 1992) and CMS_3 in sunflower (Spassova et al. 1993). The mechanism by which the *T-urf*13, *pcf* gene or other CMS genes cause pollen abortion is not yet understood. In some cases the chloroplast DNA seems to contribute to CMS (Frankel et al. 1979; Chen et al. 1990).

The analyses of cytoplasmic male sterile somatic hybrids in the genera *Nicotiana*, *Petunia*, *Solanum* and *Brassica* (Belliard et al. 1979; Boeshore et al. 1983; Boeshore et al. 1985; Barnsby et al 1987a; 1987b; Vedel et al. 1987; Jourdan et al. 1989; Landgren and Glimelius 1990; 1994; Medgyesy 1990; Pelletier 1986; 1991; Temple et al. 1992; Walters and Earle 1993; Wolters 1994) provided additional evidence for the involvement of the mitochondrial genome in CMS and gave evidence for the remarkable high recombination potential of the mt genome. Analyses of restriction fragment patterns of the somatic hybrids mtDNA showed that the mitochondrial genome differed between somatic hybrids and from the parents used in the fusion experiment. The hybrids consisted of DNA fragments from both parents in novel combinations.

Instability of sterile cytoplasm appears to be of common occurrence as is evident from frequent spontaneous or induced reversion of CMS strains to male fertility in long term callus or protoplast tissue cultures (Hanson 1984; Srivastava and Gupta 1991). The culture conditions appear to affect the stability of the plant's nuclear and organellar genomes and enhance rearrangements of the mtDNA genome (Pring et al. 1988). Fertile revertants from CMS plants, based on the loss of specific pieces of mitochondrial DNA, especially sequence alterations within the high molecular weight mitochondrial chromosome, have been observed (Cassini et al. 1977; Levings III et al. 1980; Kemble and Flavell 1982; Kemble and Shepard 1984; Shardl et al. 1985; Smith et al. 1987; Clark et al. 1988; Escote-Carlson et al. 1988; Kemble et al. 1988; Pring et al 1988; Small et al. 1988; Thomas et al. 1988 Brears et al. 1989; Hartmann et al. 1989; Brears and Lonsdale 1990; Chowdhury et al. 1990; Fauron et al. 1990; Mackenzie and Chase 1990; Saleh et al. 1990; Smith and Chowdhury 1991; Rouwendal et al. 1992). Reversions in fertile lines to CMS have also been observed as a result of

mtDNA changes (Li et al. 1988; Dudareva et al. 1990) or due to nuclear gene mutations (De Paepe et al. 1990). According to Karp and Bright (1985) and Dikalova et al. (1993) environmental stress may act as a 'genomic shock' and as an inducer of rearrangements in the mitochondrial genome during *in vitro* cell culturing. For example, under stress conditions, the generation of superoxide and hydroxyl radicals can cause double stranded breaks in the DNA.

The molecular basis of the CMS trait is still far from being understood (Vedel et al. 1994). Progress concerning mt genome expression and its role in CMS is hampered by the difficulty in transforming the higher plant mt DNA (Williams and Levings III 1992; Mackenzie et al. 1994; Vedel et al. 1994). In the future, transformation of higher plant mitochondria can be envisaged by using the particle bombardment method and protein-mediated RNA or DNA import (Vedel et al. 1994).

Restorer genes

In nearly all CMS systems, nuclear genes called "fertility restorers" (Rf genes) have been identified (Mackenzie et al. 1994). These genes enable the control of the CMS trait by restoring the male sterility. If the desired agricultural commodity is a fruit or seed, restorer genes are a necessity. Both monogenic and polygenic systems of nuclear gene CMS modification are known, and both recessive and dominant sterility modifiers have been identified (Hanson and Conde 1985). However, most restorers consist of a single dominant gene (Mackenzie et al. 1994).

The action of a restorer gene is only fragmentary understood. Two hypotheses were adopted to explain the action of restorer genes (Hanson 1991). The restorers could compensate for, or replace, the function of a disrupted mt gene, in cases where CMS results from an abnormal mt function. If CMS results from the acquisition of a novel gene that produces a toxic product, then nuclear restorer genes could act by affecting the abundance of the toxic gene or its expression. Evidence that a nuclear restorer gene affects the expression of a mitochondrial gene is often used to support the identification of the CMS-causing gene, for example, the *T-urf* and *pcf* genes (Williams and Levings III 1992). The two restorer genes in maize and the single restorer gene in petunia regulate the expression of respectively the *T-urf* and *pcf* gene and result in reduced protein levels of *T-urf* and *pcf* in all tissues (Dewey et al. 1987; Nivison and Hanson 1989;

Pruit and Hanson 1991).

In the common bean (*Phaseolus vulgaris*) the restoration of fertility by an Rf gene was permanent due to the loss of an mtDNA fragment (Mackenzie and Chase 1987; 1990).

In some CMS systems only one fertility-restoration system has been identified, although multiple fertility restorers are available for a number of cases of CMS (e.g. petunia, bean, rice, wheat, rape) (Mackenzie et al. 1994).

The wide distribution of both CMS and restorer nuclear genes may suggest a common fundamental basis for the developmental aberration.

Environmental influence

Environmental factors (i.e. temperature, photoperiod, light intensity, nutrients and pH) may influence the expression of the CMS. Kaul (1988) mentioned ten crops and some alloplasmatic combinations where the temperature influenced the expression of fertility. High temperatures and/or low temperatures may result in restoration of the fertility. Van Marrewijk (1968) performed experiments in petunia (*Petunia hybrida*) under controlled conditions and he observed an optimum temperature which results in a maximum male fertility. The photoperiod also influences microsporogenesis and tapetal development, whereas extreme photoperiodic treatments may result in male sterility. The reaction to environmental factors also depends on the genotype (Nieuwhof 1990). Temperature sensitivity in relation to the nuclear genotype has been reported for different species (Fan and Steffansson 1986). In chives (*Allium schoenoprasum*) it was possible to analyse one dominant nuclear gene which acted like a restorer gene at high temperatures of 24°C and was inactive under normal temperatures (Tatlioglu 1987). Apparently, the environmental conditions determine the activity of restorer genes.

An alternative way of inducing male sterility is the application of various phytogametocidal compounds (reviewed by Van Marrewijk 1979; Chauhan and Kinoshita 1982; Kaul 1988). These chemicals are known as male-gametocides or chemical hybridizing agents and the selective abolition of male sex is termed chemical male sterility. Kaul (1988) lists 26 different chemical gametocides. These compounds, however, do not permit the economic production of large quantities of hybrid seed (Lasa and Bosemark 1993). They are expensive, toxic to the plants, influence the female fertility, and induce male sterility for a short period only (Kaul 1988). Their application is limited further by: the necessity to determine the optimal dose for a certain plant species, a high genotypic response, the tissue specificity,

the developmental stage of the plant organ, the physiological state, environmental factors etc..

Nevertheless the gibberellic acid GA_3 and maleic hydrazide were used in the present research to induce male sterility in leek. Bolting plants from the cultivars Porino and Strata were treated with $14.4.10^{-5}$ M or $3.6.10^{-5}$ M GA_3 and $2.2.10^{-5}$ M maleic hydrazide. The chemicals were sprayed over the plant three times during the elongation of the flower stalks. These minor experiments did not result in the development of male sterile leek plants.

Pollen development

The male gametophyte completes its early development within the anther. Pollen mother cells are produced in the sporogenous tissue within the anther. The two divisions of meiosis transform these cells into haploid microspores, each pollen mother cell producing first a dyad and, after the second meiotic division, a tetrad of microspores. The tetrad and each microspore within the tetrad are surrounded by a callose wall. Upon release from the tetrad, the microspores increase rapidly in volume and undergo a change in shape. This is followed by a period of slower growth until the maximum volume of the pollen grains is reached before anthesis. Following release of microspores from the tetrads, there is an extended interphase period that terminates with a very unequal division of the microspore (mitosis), forming a vegetative cell and a generative cell (Mascarenhas 1989; Bedinger 1992). In some species the generative cell within the young pollen divides again to form two sperm cells; in many other plant species, including leek, the second mitosis takes place only after germination of the pollen tube.

The tapetum of higher plants plays a critical role in pollen development. It has long been proposed that the tapetal cells play a nutritive role for the microspores. A second tapetal function is to release the young haploid microspores from the callose wall enclosing the meiotic tetrad by the secretion of callase. The timing of callase secretion appears to be critical for normal pollen development. A third proposed role for the tapetal cells is the production of precursors for the biosynthesis of the outer pollen wall, or exine.

Degeneration of the pollen is most often caused by the abnormal behaviour of the tapetum (Pearson 1981). A premature breakdown of the tapetum has been considered as the main cause of pollen sterility in higher plants. This is evidenced by examples from many plant species, in all of

which tapetum is persistent and the microspores abort just before or just after release from the tetrads (Kaul 1988). The critical nature of this tissue has been demonstrated in transgenic plants (Mariani et al. 1990). In the majority of cases of both NMS and CMS with abnormal tapetum, breakdown of the tapetum occurs after tetrad formation and during microspore development. However, visible tapetal anomalies may not be a necessary prerequisite for male sterility.

Microscopic evidence suggests that mitochondrial activity is important in the tapetal cell layer. During early development of normal maize anthers, mitochondria increase in number about 20 times in sporogenous tissue and 40 times in tapetal cells (Warmke and Lee 1978). The capacity to study gene expression and enzyme activities in specific cell types, such as tapetal cells, would benefit to the understanding of CMS (Williams and Levings III 1992).

Staining methods

Staining methods are used mostly to estimate male fertility. The ultimate functional test in this respect ought to be the fertilising capacity as measured by the seed set. To measure this, involves a considerable investment of time and is, practically speaking, not feasible (Heslop-Harrison et al. 1984). Heslop-Harrison et al. (loc.cit.) distinguished four different tests for estimating the pollen quality: (1) *in vitro* germination test, (2) stainability test (aceto-carmine, lactophenol-acid fuchsin), (3) enzyme test (tetrazolium chloride), and (4) fluorochromatic (FCR) test. Comparison of the results in these four tests indicated that the staining procedures provided the poorest guide to germinability, whereas the FCR procedure and the *in vitro* test gave the best guide.

Staining with aceto-carmine, which involves staining of the cytoplasm and nucleus, is one of the earliest tests for checking the pollen quality. Pollen that can not be stained has no cytoplasm and is thus not vital. According to Hoekstra et al. (1989) this test can be performed in cases were there is an increased chance for male sterility, indicating that this test is better suitable for checking on male sterility than on male fertility. The test results in an under-estimation of the actual cases of male sterility, since this method only tests the presence of a cytoplasm and nucleus and not the viability of the pollen. However, there is the absolute guarantee that male sterile plants, exhibiting pollen without cytoplasm and/or nucleus, will be identified by means of this test.

Preliminary experiments

Male sterility types in leek

Male sterile (MS) flowers in leek could visually be distinguished from male fertiles (MF) by differences in the flower and anther morphology. In our experiments, all MS leek flowers had a white petal colour, with sometimes a pink shine as a result of anthocyan expression. MF anthers were thick and swollen with long filaments lifting the anthers outside the flower. The anthers of MS plants were shrivelled and small as compared to anthers of MF plants (Fig.1). However, in some cases the anthers of MS plants were similar in appearance to the anthers of MF plants and the male sterility could only be detected by staining the pollen of every plant with aceto-carmine. The anther colour of MS plants varied from almost translucent white or yellow in an early developmental stage, to dark brown in a later stage. The filaments of MS anthers sometimes were very short, resulting in anthers remaining inside the flower. Occasionally, the anthers were completely absent or only the filaments were present (Fig.1).

Three morphologically distinct 'sporogenous' pollen aberration types were detected at the microscopic level in MS leek plants of cultivar Porino, after staining the pollen with aceto-carmine (Fig 2). The first aberration type resulted from an inability to release the microspores from the tetrads during the tetrad stage. Instead of this, tetrads stuck together (clumped) and finally degenerated. This type was found among about 20% of the observed MS plants. In the second type, the degeneration of the microspores began during the maturation period of the microspores, after the release from the tetrads. The cytoplasm of the microspores disappeared completely and the microspores collapsed. This type was most frequently observed (76%). In a very few cases (4%) the pollen was completely absent at the time of anther dehiscence. Pollen from MF plants, on the contrary, showed an oval shape, whereas the cytoplasm and two nuclei stained red with aceto-carmine. A histological examination of the tapetum behaviour in male sterile and male fertile leek plants is desirable to reveal the causes of pollen abortion.

Holford et al. (1991) reported three types of abnormal tapetal behaviour in male sterile onion anthers. However, these abnormalities were always observed after the release from the tetrads.

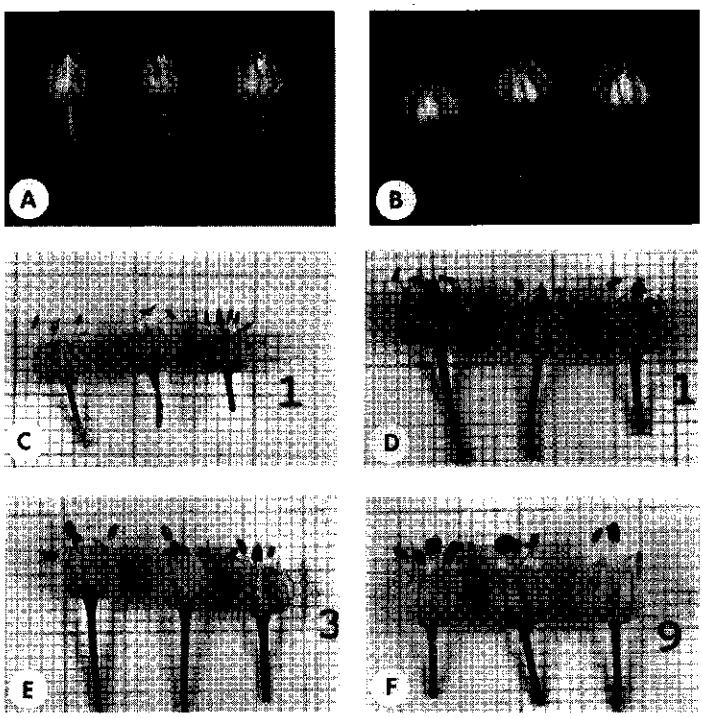

Fig. 1. Differences in anther morphology of male sterile (MS) and male fertile (MF) flowers of leek; **A.** MS flower; anthers completely absent; **B, C, D.** MS flowers with small, brown and shrivelled anthers, anthers remaining inside flower (**B**) or anthers lifted outside flower (**C, D**); **E.** MS flower with normal looking anthers; **F.** MF flower.

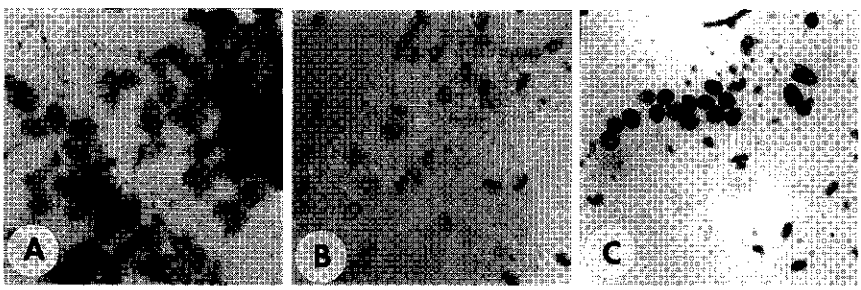

Fig. 2. Light microscope views of male sterile (MS) and male fertile (MF) pollen types of leek, after staining with aceto-carmine acid (2%); **A.** pollen of MS plant in tetrad configuration (type 1, *x*100); **B.** pollen of MS plant empty and collapsed (type 2, *x*100); **C.** stained pollen of MF plant (*x*100) .

Male sterility experiments in leek

Based on the origin of CMS, two different approaches can be followed to obtain CMS in leek: (1) searching for spontaneous (autoplasmic) CMS, or creating a new form of autoplasmic CMS in leek by mutation breeding, and (2) creating a form of alloplasmic CMS by intra- or interspecific hybridization. The results from the efforts to obtain CMS via both ways will be discussed.

Spontaneous male sterility in leek

Naturally occurring forms of nuclear (NMS) and cytoplasmic male sterility (CMS) have been found in many cultivated species (reviewed by Kaul 1988). CMS types discovered in the wild, or segregated out of cultivars were rapidly moved into seed production programs as was the case in beet, onion, carrot, corn, sorghum, radish, and rice (Pearson 1981). Concerning leek, it can not be excluded that a form of CMS is already present within the large *Allium ampeloprasum* complex and an extensive survey of seed production fields of different leek cultivars, as recommended by Kampe (1980), seemed worthwhile for leek.

Seed production fields of leek in Italy were examined for apparently spontaneously occurring male sterile (MS) plants (Table 1). These plants were traced by visually examining the flowers for anther anomalies and subsequently staining the pollen with a solution of 2% aceto-carmine. No distinction in male sterility type was made. MS plants derived from the different cultivars were 'open'-pollinated in the field by male fertile (MF) plants of the same cultivars. The obtained half-sib (HS) progenies were examined in the following year in the Netherlands for male sterility (Table 2). The MS plants within these HS-families were cross-pollinated with MF plants of the same HS family and their full-sib (FS) progenies were examined subsequently in the following year (Table 3).

A low total percentage (0.02%) of MS plants was discovered among the different cultivars in the seed production fields. Differences between cultivars in the percentage of MS plants were observed. The cultivar Arkansas showed the highest percentage (0.05%) of MS plants. These differences in frequencies probably resulted from overlooking MS plants in the field. A HS-family with 100% MS plants was not observed. Nine progenies of the collected MS plants, however, showed relatively high percentages of MS plants

(Table 2). The remaining 24 progenies were completely MF.

Table 1. Number and percentages of observed male sterile (MS) plants of leek cultivars in seed production fields in Italy.

Cultivar Number	Cultivar	Plants Examined Number	MS Plants Number	(%)
983	Romil	59 000	11	(0.02)
1031	Argenta	69 700	3	(0.00)
1035	Arkansas	31 700	16	(0.05)
1036	Argenta	11 500	2	(0.02)
1046	Vincent	29 000	3	(0.01)
1048	Poriver	36 000	3	(0.01)
1051	Portant	19 260	6	(0.03)
Total		256 220	44	(0.02)

Schweisguth (1970) also reported the presence of MS plants in selfings and open-pollinations. MS plants were found in a frequency of 10^{-4} in 'open' pollinated leek cultivars. The male sterility was found to be controlled in some cases by a monogenic recessive gene, while in other cases two, three

Table 2. Number and percentages of observed male sterile (MS) plants in half-sib (HS) progenies derived from MS plants of different leek cultivars from seed production fields in Italy.

HS-family Number	Cultivar	Plants Examined Number	MS Plants Number	(%)
983-22	Romil	43	5	(11)
983-53	Romil	4	2	(50)
983-55	Romil	17	2	(12)
983-68	Romil	5	1	(20)
983-87	Romil	31	2	(7)
1031-21	Argenta	83	5	(6)
1031-82	Argenta	94	6	(5)
1035-05	Arkansas	82	6	(7)
1035-30	Arkansas	72	2	(3)

or more genes seemed to be responsible. Rauber (1989) also reported that male sterility was controlled by a monogenic recessive inheritance. In some cases, based on segregation ratios, two genes seemed to control the male sterility trait.

The low percentages of MS plants can be expected, because MS plants have, in theory, a selection disadvantage in nature, because their genetic material can only be distributed via the egg cells and not via the pollen to their progenies. This selection disadvantage results, in theory, in the disappearance of MS plants in a population. However, heterosis caused by outbreeding and probably resulting in a higher vigour and more seed production of the MS plant might compensate for this disadvantage.

The MS plants in the HS-families are the result of a cross between a MS (♀) plant and genotypically different MF (♂) plants (Fig. 3). If we assume a model for monogenic recessive inheritance for the MS trait in our experiments, than the frequency of MS plants in the progeny of a MS plant, after open-pollination with genotypically different MF (♂) plants, depends on the frequency of 'msms' gametes that is produced by the pollinators (♂),

Fig. 3. Model for monogenic inheritance of the MS trait in leek. MS plants derived from seed production fields and subsequent crossing experiments.

MS. . . = MF genotype
msmsmsms = MS genotype
♀,♂ = resp. female, male
x = cross
\# = not analysed
o = selfing

♀ msmsmsms x ♂ MS....

↓

MSMSmsms / Msmsmsms / msmsmsms

♀ msmsmsms	x ♂ MSMSmsms	♀ msmsmsms	x ♂ Msmsmsms
	o		o
↓	↓	↓	↓
1/6 MSMSmsms	35/36 MS. . .	1/2 Msmsmsms	3/4 Ms. . .
4/6 Msmsmsms	1/36 msmsmsms	1/2 msmsmsms	1/4 msmsmsms
1/6 msmsmsms			

because only a combination of 'msms' ♀ gametes and 'msms' ♂ gametes results in MS plants ('msmsmsms'). The frequency of 'msms' gametes, in turn depends on the frequency of 'MSMSmsms' and' MSmsmsms' genotypes ('msmsmsms' is male sterile) in a population of leek plants. Based on the average frequency of 2.10^{-4} MS ('msmsmsms') plants in our leek populations, 20% selfing, and a Hardy-Weinberg equilibrium for the MS trait, the frequencies of the 'msms' gametes can be calculated. This calculation results in an expected frequency of $2.5.10^{-3}$ (0.25%) of MS ('msmsmsms') plants in the progeny of MS (♀) plants. It is obvious from table 2 that the observed frequency of MS plants in these progenies exceeds the theoretic expected frequency. The surplus of MS plants indicates that there was a surplus of 'msms' gametes and therefore a surplus of 'MSMSmsms' and 'MSmsmsms' genotypes in the pollinator population. This result can be explained by an underestimation of the frequency of MS plants in the population, and thus by overlooking MS plants in the field or by an overestimation of the MS plants in the progenies. Theoretical calculations (20% selfing and Hardy-Weinberg equilibrium) show that the high percentage of MS plants in the HS progenies can be explained by supposing approximately 1% of MS plants ('msmsmsms') in the original leek populations.

The MS plants in the HS-progenies were crossed with MF plants of the same HS-progenies (sib-crossing) (Fig. 3), in order to find out how many genes controlled the MS trait. The MF ♂ plants only can be presented, assuming a model of monogenic inheritance for the MS trait, by either the genotypes 'MSMSmsms' (duplex) or 'MSmsmsms' (simplex) (Fig 4). Both the progenies of the MS ♀ plant and MF ♂ plant (selfing or I_1) were examined for male sterility. These sib-crosses resulted in a specific number of MS plants in the progenies of both the MS (♀) plant and the MF (♂) plant (Table 3). The genotype (duplex or simplex) of the ♂ parent can be determined by the segregation ratios of MS plants in both the progenies. A chi-square analysis, therefore, was performed on the ♀ and ♂ progenies to test whether the observed number of MS plants corresponded with the expected segregations for a monogenic inheritance (i.e., 1:1 resp. 1:5 for the MS progeny and 1:3 resp. 1:35 for the I_1), assuming resp. a simplex or duplex ♂ parent. The genotype of the ♂ parent could be determined after some crosses. The genotype of some ♂ parents did not match with the segregation ratios in the progenies of the ♀ parent, due to higher percentages of MS plants in the I_1 progenies. The limited number of flowering plants was a major obstacle in determining the genotype of both parents. The surplus of MS plants in some

Table 3. Numbers and percentages of observed male sterile (MS) plants in full-sib (FS) progenies of leek derived after cross-pollination of MS plants with male fertile plants of the same half-sib.

	Progenies of MS ♀			Progenies of MF ♂			Genotypes parents	
FS-Number	Observed MF:MS	Expected[1] MF:MS 1:1	Expected[1] MF:MS 1:5	Observed MF:MS	Expected[1] MF:MS 1:3	Expected[1] MF:MS 1:35	MS ♀	MF ♂
983-22-01	13 : 2	-	+[2]	11 : 2	+	-	msmsmsms	MSmsmsms
983-22-03	92 : 5	-	-	7 : 1	+	+	msmsmsms	?
983-55-01	85 : 2	-	-	- : -			msmsmsms	?
983-87-02	5 :15	+	-	5 : 3	+	-	msmsmsms	MSmsmsms
1031-21-01	24 : 3	-	+	- : -			msmsmsms	?
1031-21-02	54 :20	-	-	2 : 3	+	-	msmsmsms	MSmsmsms?
1031-21-03	18 : 2	-	+	13 : 0	-	+	msmsmsms	
1031-82-02	37 : 3	-	+	2 : 0	+	+	msmsmsms	
1031-82-03	10 : 5	+	-	1 : 1	+	-	msmsmsms	MSmsmsms
1031-82-04	37 : 2	-	+	8 : 0	+	+	msmsmsms	
1031-82-05	16 : 8	+	-	5 : 3	+	-	msmsmsms	MSmsmsms
1035-05-02	40 : 4	-	+	- : -			msmsmsms	?
1035-05-03	16 : 1	-	+	- : -			msmsmsms	?
1035-05-04	27 : 2	-	+	4 : 3	+	-	msmsmsms	?
1035-05-07	36 :15	-	-	31 : 0	-	+	msmsmsms	
1035-30-01	32 :14	-	-	8 : 3	+	-	msmsmsms	MSmsmsms

[1] Chi-square analysis with $P \leq 0.05$

[2] "+" indicates that the observed frequency corresponds with the expected frequency

I_1 progenies can be caused by inbreeding depression. Based on these results, a monogenic inheritance can be postulated for the genetic control of the MS trait in most of the progenies (Table 3).

A monogenic (NMS) inheritance for the MS trait in leek was also observed by Schweisguth (1970) and Rauber (1989). However, the acceptance of a hypothesis of nuclear inheritance does not automatically rule out the possibility of a nucleo-cytoplasmic interaction. Rauber (1989) already suggested that the MS trait may be controlled by a restorer gene acting upon a sterilizing cytoplasmic background, but she could not prove this assumption. This hypothesis, however, can explain the high frequency of MS plants in some of our progenies. The following genotypes and two cytoplasms can be distinguished in a theoretical model:

(S_1) Rf_1. . .	=male fertile	(S_2) Rf_1 . . .	= male fertile
(S_1) $rf_1rf_1rf_1rf_1$	=male sterile	(S_2) $rf_1rf_1rf_1rf_1$	= male fertile

This situation is comparable to NMS, because the genotype (S_1) $rf_1rf_1rf_1rf_1$ results in the MS phenotype. In the additional S_2 cytoplasm (N.B. some people prefer the term "N" or "F" cytoplasm) the Rf_1 gene has no

phenotypic consequence, because it specifically acts upon the S_1 cytoplasm, and the additional S_2 cytoplasm gives the recessive rf_1 gene the opportunity to accumulate in a higher (random mating) frequency. Thus a nucleo-cytoplasmic (CMS) inheritance may well serve to explain the male sterility trait in leek; especially our unexpected high frequency of MS plants in the progenies from Italy. Two different cytoplasms already have been distinguished in leek (Chapter 3). The exploitation of this system depends on the identification of the maintainer genotype (S_2) $rf_1rf_1rf_1rf_1$.

Intra- and interspecific crosses

The majority of the known cases of cytoplasmic male sterility have resulted from the combination of a nucleus with an alien cytoplasm (reviewed by Edwardson 1970; Kaul 1988). This cytoplasm may have evolved independently of the nucleus in a separate species or in another population of the same species.

The generally applied method for introducing alien cytoplasms into cultivated species consists of a sexual cross using the cytoplasm donor as the

female, followed by several back-crosses with the cultivated species as the pollinator.

Within the large *Allium* genus, the common onion (*Allium cepa*) would be a good candidate for the donation of the cytoplasm to leek. In onion, a CMS trait was discovered by Monosmith (1926) and has since been used for commercial hybrid onion production (Jones and Emsweller 1937). Crossing experiments to transfer the trait from onion to leek were performed by Dehne et al. (1979), but these experiments were not successful. Hybrid plants from a cross between *A. chinense* and *A. ampeloprasum* (great headed garlic) were obtained by using ovary culture (Nomura et al. 1994).

In the present research, crosses were made, within the large *Allium ampeloprasum* complex, with wild *A. ampeloprasum*, kurrat (syn. *A. kurrat*) and *A. commutatum* plants as female (♀) parents. Individual flowers (± 17 per plant) were emasculated and the remaining flowers cut off, in order to be used as female parents. Developing new flowers were removed as soon as they were observed. Leek plants of the cultivar Porino were used as the male (♂) parents. The plants were put in an isolation cage and blow flies were used for performing the cross.

Different crosses did not result in seed set, whereas in some others the seeds did not contain an embryo or failed to germinate (Table 4). It was remarkable that all established hybrid progenies were completely and highly male fertile. All the progenies showed a 4*x* ploidy level (flow cytometry analysis). The male fertility already implicated that the difference in cytoplasm was not large enough for creating a male sterility trait in the hybrid. However, there was still a possibility that both cytoplasms were controlled by nuclear fertility restorer genes and that the fertility in the F_1 resulted from specific restorer genes related to the donor cytoplasm. Therefore, several plants of the F_1 hybrid progenies either were backcrossed with 'Porino' plants or selfed.

Fixed frequencies of MS plants were expected in both the backcross progenies and selfings, and chi-square tests were used to determine whether the observed frequencies corresponded with the expected frequencies. However, the observed frequencies of male sterile plants in the backcross generations and selfings were far below the expected frequencies, and therefore the assumption of a nucleo-cytoplasmic inheritance had to be rejected.

Table 4. Seed set, number of seedlings, number of flowering plants, ploidy level and observed number of male sterile (MS) in progenies of backcrosses and selfings after interspecific crosses of leek with relatives from the *A. ampeloprasum* complex.

♀ Parent	F_1					
Number plants	Group	Seed set	Seedlings Number	Flowering plants Number	Ploidy[1]	MS
90-01	ampeloprasum	yes	44	37	4*x*	0
90-02	ampeloprasum	yes	0			
90-03	ampeloprasum	yes	2	0	4*x*	0
90-04	kurrat	no				
90-05	ampeloprasum	yes	5	0	4*x*	0
90-07	ampeloprasum	yes	0			
90-08	ampeloprasum	yes	0			
90-10	ampeloprasum	yes	0			
90-11	ampeloprasum	yes	0			
90-12	ampeloprasum	no				
90-14	ampeloprasum	yes	13	10	4*x*	0
90-15	ampeloprasum	yes	2	0	4*x*	0
90-16	ampeloprasum	no				
90-17	commutatum	no				
90-18	ampeloprasum	yes	0			
90-19	ampeloprasum	yes	2	0		
90-20	ampeloprasum	yes	0			
90-21	ampeloprasum	no				
90-22	ampeloprasum	no				
90-23	ampeloprasum	yes	0			
90-24	ampeloprasum	yes	15	0	4*x*	0
90-25	ampeloprasum	yes	0			
90-26	ampeloprasum	yes	0			
90-27	ampeloprasum	yes	0			
90-28	ampeloprasum	no				
90-29	ampeloprasum	yes	0			
90-30	commutatum	yes	4	2	4*x*	0
90-31	ampeloprasum	yes	11	9	4*x*	0
90-32	kurrat	yes	4	0	4*x*	0
90-33	ampeloprasum	yes	0			
90-34	ampeloprasum	yes	0			
90-35	ampeloprasum	yes	0			

Differences in ploidy level are a major obstacle in performing crosses between different species, and were the reason for the failure of some of our crossing experiments. Wild *ampeloprasum* shows various levels of polyploidy, 2*x*, 3*x*, 4*x*, 5*x*, 6*x*, 7*x*. However, tetraploid and hexaploid are predominant in wild populations. The failure to set seed in some of the crosses with wild *ampeloprasum* material can be ascribed to this imbalance in ploidy level. Leek and kurrat are 4*x*, whereas *A. commutatum* is 2*x* or 3*x*. It was remarkable that one cross with *A. commutatum* gave viable seed.

Intraspecific crosses between the different groups within the *A. ampeloprasum* complex are up till now an unexploited topic, that can give further insight in the relationship between the different groups, and also could be of great value for practical breeding through the establishment of a CMS trait in the cultivated leek. Crossing experiments within the *A. ampeloprasum* complex should therefore be encouraged. A candidate for intraspecific crosses with the cultivated leek is *A. polyanthum*. This "species" provides a pool of interesting genetic characteristics such as male sterility and differential attraction to phytophagous consumers (Boscher et al. 1989).

Maybe there is even a possibility to introduce CMS from the onion (*A. cepa)* by performing a cross between the interspecific onion hybrid (*A. cepa x A. fistulosum*) (4x) and leek. Embryo rescue techniques could be employed to optimize the chances of obtaining viable hybrid plants.

Sometimes the relationship between *A. ampeloprasum* and garlic (*A. sativum*) is discussed because of morphological resemblance. From a viewpoint of karyosystematics, Tarrasova (1987) suggested a possibility of evolution from *A. sativum* through *A. ampeloprasum* to *A. porrum*. Etoh et al. (1992) reported that leek and garlic showed similarity in peroxidase isozymes and concluded that leek and garlic have a common ancestor.

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CHAPTER 3

Induction of (cytoplasmic) male sterility in leek (*Allium ampeloprasum* L.) by chemical mutagens

B.C.H.J. Silvertand[1], C. Kik[2] and A.M. Van Harten[1]

[1] The Graduate School of Experimental Plant Sciences, Department of Plant Breeding, Wageningen Agricultural University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands
[2] Centre for Plant Breeding and Reproduction Research (CPRO-DLO), P.O. Box 16, 6700 AA Wageningen, The Netherlands

Summary. Attempts have been made to induce cytoplasmic male sterility in leek (*Allium ampeloprasum* L.) using N-nitroso-N-methyl urea (NMU). In preliminary experiments the mutagenic activity of different chemical mutagens was compared and the chemical NMU was selected for large mutation experiments using seeds of cultivar Porino. NMU treatments resulted in a poor seedling emergence, a high percentage of chlorophyl deficient seedlings and a high percentage of male sterile plants in the M_1 generation. M_1 survival rates were very low. Only 16% of the treated seeds developed into flowering plants. All NMU treatments resulted in a reduced seedling emergence in the M_2, whereas treatments higher than 10 mM NMU produced increased percentages of chlorophyl deficient seedlings. In the M_2 generation, plants with yellow/white stripes were observed, which showed a non-Mendelian inheritance. Percentage of male sterile plants in the M_2 varied between 0.2% and 0.5% and did not differ from the control. Eight half-sib M_2 families with high percentages of male sterile plants were found. Progenies (M_3) of these families contained a very low percentage of male sterile plants. Mitochondrial DNA analysis using three different restriction enzymes revealed the existence of two mitochondrial types within the cultivar Porino. No additional mtDNA variation was found in the induced male sterile plants. Both crossing experiments and restriction analyses indicate that the induced male sterility was determined by nuclear genes, rather than by an interaction between nuclear and cytoplasmic genes.

Introduction

Leek is a mainly outbreeding autotetraploid ($2n=4x=32$) species, belonging to the diverse species complex *Allium ampeloprasum* L. (Van der Meer and Hanelt 1990). It is mainly grown in France, Belgium, The Netherlands and United Kingdom, for its edible pseudostem formed by the leaf sheaths. Leek cultivars suffer from severe inbreeding depression (Berninger and Buret 1967; Gray and Steckel 1986), and mass and/or family selection have been the main methods of cultivar improvement. As a consequence of these breeding methods, leek cultivars are not uniform or stable. Lack of uniformity and severe disease problems reduce the yield and quality of the product at harvest. The most promising system for improvement of uniformity, yield and disease resistance seems to be hybrid breeding (Schweisguth 1970; Kampe 1980; Smith and Crowther not published). In general, F_1 hybrids may show a high amount of heterosis, phenotypic stability and uniformity (Wricke 1989). A prerequisite, however, for F_1 hybrid breeding is the availability of male sterile

lines. Male sterile plants based on nuclear recessive genes have been found within leek cultivars (Schweisguth 1970; Rauber 1989). Since male sterile plants must be produced by crossing male steriles with heterozygous male fertiles, the progeny is partially male fertile and this is a great disadvantage, unless closely-linked markers are available. Therefore, the most promising genetic system for hybrid seed production in leek seems to be cytoplasmic male sterility (CMS). Unfortunately, no source of CMS is yet available in leek or related forms of *A. ampeloprasum*. CMS is a valuable tool for plant breeders which can arise spontaneously, through inter- or intraspecific crosses, protoplast fusions, after mutagenic treatment and by passage through tissue culture (Williams and Levings III 1992).

CMS has been induced using chemical or physical mutagens in fertile cytoplasms in several plant species (Kaul 1988). From a number of chemical mutagens it is known that they also act upon mitochondria and chloroplasts.

The mutagenic activity of Ethidiumbromide (EB) was investigated primarily in unicellular organisms and in cultures of animal cells and it was shown that it effectively induces mutations affecting the mitochondria and chloroplasts (Mayer and Simpson 1969; Alexander et al. 1974; Levy and Ashri 1975). EB induced cytoplasmic mutations in yeast (Slonimski et al. 1968; Nagley and Linnane 1972; Uchida and Suda 1973) and CMS in pearl millet (Burton and Hanna 1976), sugar beets (Kinoshita et al. 1982) and maize (Chaudhary and Minocha 1989). Ethyl methane sulphonate (EMS) is generaly considered as a very effective mutagen (Kawai 1969; Kaul and Bhan 1977). CMS was induced by EMS in the N-cytoplasm of barley (Favret and Ryan 1964), sugar beet (Kinoshita et al. 1979) and rice (Mallick 1980; Minocha and Gupta 1988). *N*-nitroso-*N*-methyl-urea (NMU) is also a very efficient mutagen; it induced nuclear gene and chromosome mutations and plastome mutations (Kawai 1969; Razoriteleva et al. 1970; Nilan 1972; Pohlheim and Beger 1974; Hagemann 1976, 1979, 1982; Pohlheim 1981; Hosticka and Hanson 1984; Fluhr et al. 1985; Davidson et al 1987; Walters et al. 1990; Sears and Sokalski 1991). Malinovskii et al. (1973) reported induction of CMS in sorghum by using NMU. Sodium azide (SA) is mutagenic in many plant species (Kleinhofs et al. 1978). It has the characteristic of producing primarily, if not solely, point mutations (base substitutions) with no evidence of chromosomal aberrations (Rines 1985). In barley it induced reduction of seed germination, seedling growth and increased male sterility. Streptomycin (STR) is used as a common antibiotic in tissue culture systems. It acted as a mutagen for cytoplasmic genes in *Chlamydomonas* (Sager 1972). Burton and Hanna (1982) obtained cytoplasmic mutants in pearl millet,

Kinoshita et al. (1982), Mikami et al. (1980) in sugar beets and Jan and Rutger (1988) in sunflower after treatment of seeds with streptomycin. Petrov et al. (1971) patented a method for inducing CMS in maize using streptomycin.

In this study we focussed our attention on the induction of male sterility by means of chemical mutagenesis.

Materials and methods

Chemical mutagens

The chemical mutagens EB (2,7-diamino-10-ethyl-9-phenylphenantridium bromide)(Boom BV), EMS (Eastman-Kodak Co), NMU (Roth BV), SA (Boom BV) and SM (Boom BV) were used.

Plant material; mutagenic treatments

Experiments started in 1988 and continued until 1993. The plant material consisted of seeds of the leek cultivar Porino (Nunhems Zaden, Haelen, The Netherlands). Seeds were put in paper teabags (280 seeds per bag) and rinsed for 16 h in running tapwater at room temperature (cf. Savin et al. 1968). After pre-soaking, the seed bags were transferred to glass jars containing freshly prepared mutagenic solutions (for concentrations see results). The seeds were submerged for 2 h with air bubbled into the solution at room temperature in the dark. The sodium azide solutions were buffered at pH 3.0 with a 0.1 M potassium phosphate buffer. Seeds were washed in running tapwater for 8 h after the treatments and subsequently air-dried for 4 h. Immediately after drying, the seeds were sown, individually with a pincet, in plastic boxes (280 seeds per box) . The boxes were placed in a greenhouse and covered with plastic during one week which facilitates germination.

Growing of the M_1, M_2 and M_3 generation

The M_1 was repeatedly examined and percentages of emerging seedlings and chlorophyl deficient seedlings were recorded. M_1 plants were raised in a greenhouse for 12 weeks and after this period transplanted to the field in 1989. For natural vernalization, leek plants remained on the field during the winter period. Flowering M_1 plants were open-pollinated and seeds were harvested individually per plant in 1990. The obtained half-sib M_2 progenies

were sown in March 1991 and transplanted to a trial field in the autumn, where they were kept during the following winter period. Individually flowering M_2 plants were visually examined for male sterility in 1992. Male sterility was confirmed by crushing the individual anthers on glass slides and staining the pollen in a 2% aceto-carmine acid solution. Selected male sterile M_2 plants were isolated from fertile M_2 plants and open-pollinated by male fertile plants from different leek cultivars. The half-sib M_3 progenies, derived from the male sterile plants, were sown immediately and flowering was induced artificially after a period of cold storage (12 weeks at 9°C and 16 h light).

CpDNA and mtDNA analysis

Mitochondrial DNA was isolated from the white part of the pseudostem (100 g) and chloroplast DNA from well developed leaves (10 g), according to the procedure described by Wilson and Chourey (1984) and revised by Bookjans et al. (1984). MtDNA samples were digested with the enzymes *Eco*RI, *Xho*I and *Bam*HI. CpDNA was restricted only by *Eco*RI. Digests were separated by electrophoresis in a 0.8% agarose gel. Lambda DNA was digested with *Bst*EII and used as molecular weight marker. The electrophoresis was run for 16 h at 25 Volts (approx. 20 mA). Gels were stained with ethidium bromide for 30 min and photographed with polaroid 665 film under UV light.

Experiments and Statistical analysis

The total number of emerging seedlings and number of chlorophyl deficient seedlings per treatment was recorded 21 days, after seed treatment with chemical mutagens, and percentages were calculated. No normal distribution of the data was found in our experiments. Therefore, results were analysed using the Mann-Whitney 'U' test with $P \leq 0.05$.

Results

Preliminary experiments

In the initial experiments five different chemical mutagens in various concentrations were tested for their mutagenic effectiveness in order to select

Table 1. Effects of different chemical mutagens on seed germination and chlorophyl deficiencies (*albina*) in the M_1 generation of leek cv. Porino, 21 days after seed treatment. Average percentages for seed germination and *albina* seedlings for six replications.

Mutagen[1]	Conc. (mM)	Seeds treated Number	Seeds germinated %	*albina* seedlings %
EB	0.3	750	80	4
	0.7	1 500	76	4
	1.4	1 500	68	5
	2.8	1 500	59	5
	5.6	1 500	51	4
	11.2	750	30	4
EMS	25	750	83	4
	50	750	81	3
	100	750	81	2
	200	750	84	4
	400	750	86	2
NMU	5	750	87	4
	7.5	750	88	7
	10	1 500	84	13
	12.5	1 500	81	13
	15	1 500	79	15
	17.5	750	74	16
	20	750	73	17
	22.5	750	63	17
SA	50	750	85	3
	100	750	85	4
	200	750	81	3
	400	750	86	3
	800	750	82	3
STR	0.12	750	89	5
	0.24	1 500	85	6
	0.48	1 500	84	4
	0.96	1 500	82	5
	1.92	1 500	84	7
	3.84	750	78	5
control		2500	92	4

[1] EB = ethidium bromide; EMS = ethyl methane sulphonate; NMU = N-nitroso-N-methyl-urea; SA = sodium azide; STR = streptomycin

the most suitable mutagen for treating leek seeds (Table 1). The occurrence of chlorophyl deficient seedlings (*albinas* cf. Gustafsson 1940) in the M_1 was used as indicator for the effectiveness of the different mutagens. Increased concentrations of EB resulted in a decrease of the germination rates (Table 1). A significant ($P \leq 0.05$) negative correlation coefficient ($r=-0.72$) was found between the EB concentration and the percentage of germinated seeds. Concentrations higher than 5.6 mM gave less than 50% of germinating seeds. EMS resulted in a slightly increased seed germination (Table 1). Increased NMU concentrations caused a significant decrease ($r=-0.78$, $P \leq 0.05$) in the seed germination and a significant increase ($r=0.60$, $P \leq 0.05$) in chlorophyl deficient seedlings (Table 1). The mutagen SA did not affect the germination rates, whereas STR induced a small decline in germination at concentrations above 0.96 mM. The control showed, as expected, the highest germination rate.

As the mutagenic effectiveness may be indicated by a reduction of germination or seedling growth (Gaul 1959; Ehrenberg 1971) and by an increasing number of chlorophyl deficient seedlings in the M_1 generation (Gaul 1970), NMU was chosen as the mutagen which may maximize the chances for inducing mutations in leek.

NMU mutation induction experiments

Based on the initial mutation experiments the chemical mutagen NMU was selected for a mutation experiment in 1989. The leek seeds were soaked in 10 mM or 12.5 mM for 0.5 or 1 h. Table 2 shows that different NMU treatments did not result in a reduced seed germination. The two 1 h treatments exhibited the highest loss in surviving and flowering plants. Relatively high percentages of male sterile plants were observed in the M_1 generation for all treatments, as compared to the control. The one hour treatments resulted in the highest percentages of male sterile plants. Male sterile plants exhibited small, lanceate, shriveled and brownish or white anthers. The male sterile plants often were misformed and did not set seed (female sterility). The flowering M_1 plants were open-pollinated and seed was harvested separately per M_1 plant in September 1990. The number of harvested seeds per plant varied between 0 and more than 1000.

M_2 progenies from selected male sterile M_1 plants were sown in the greenhouse in October 1990 and flowered in July 1991, after a period of artificial vernalization. Of the 412 selected male sterile M_1 plants, only 59

Table 2. Effect of different NMU treatments on the number and percentage of germinating seeds (21 days after sowing), flowering plants and male sterile (MS) plants in the M_1 generation of leek cv. Porino.

Mutagen	Conc. (mM)	Time (h)	Seeds treated Number	Seeds germinated Number (%)	Plants flowering Number (%)	Plants male sterile Number(%)
NMU	10	0.5	5 508	4 012 (73)	994 (25)	98 (10)
NMU	10	1.0	5 184	3 911 (75)	745 (19)	127 (17)
NMU	12.5	0.5	5 184	4 133 (80)	1 167 (28)	102 (9)
NMU	12.5	1.0	4 536	3 456 (76)	260 (8)	85 (33)
control			4 536	3 158 (70)	1 106 (35)	7 (1)

Table 3. Mutagen treatment, number of surviving M_2 HS-families and pooled numbers of flowering plants and male sterile plants per treatment of leek, observed in M_2 progenies from selected male sterile (MS) M_1 plants of cv. Porino.

Mutagen	Conc. (mM)	Time (h)	Number	HS families Number	Plants total Number	Plants flowering Number	Plants male sterile
NMU	10	0.5		23	490	209	3
NMU	10	1.0		23	383	106	1
NMU	12.5	0.5		11	303	67	0
NMU	12.5	1.0		2	83	27	3
control				1	50	29	0

progenies (14%) were obtained (Table 3). All these 59 progenies either comprised of completely male fertile plants or yielded a very low number of male sterile plants.

The 3166 half-sib M_2 families (± 60 000 plants) from fertile M_1 plants were sown in the field and subsequently transplanted to a large trial field in 1991. Seed samples of these M_2 families also were sown in the laboratorium and percentages of germinating seeds and *albina* seedlings per treatment determined (Table 4).

The percentages of germinating seeds for all treatments, including the control, were very low. All treatments significantly showed a reduced germination percentage as compared to the control. The negative effect of the mutagenic treatment on seed germination in the M_2 generation is demonstrated. The percentage of *albina* seedlings induced by the 10 mM NMU treatments was significantly higher, compared to the other treatments.

Table 4. Effect of different NMU treatments on the number and percentage of germinating seeds and *albina* seedlings in the M_2 generation of leek cv. Porino.

Mutagen	Conc. (mM)	Time (h)	Seeds sown Number	Seeds germinated Number (%)	*albina* seedlings Number (%)
NMU	10	0.5	19 700	8 355 (42) a[1]	67 (0.8) a[1]
NMU	10	1.0	4 550	1 194 (26) b	15 (1.3) a
NMU	12.5	0.5	13 650	3 735 (27) b	11 (0.3) b
NMU	12.5	1.0	2 250	321 (14) c	1 (0.3) b
control			1 450	805 (55) d	2 (0.2) b

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

In the trial field, only 1 464 of the 3 166 half-sib families produced germinating seedlings or viable plantlets. Within M_2 families, plants with white/green or yellow/green stripes (*striatas* cf. Gustafsson 1940) and dwarf plants, often exhibiting a disturbed growth behaviour, were observed and recorded (Table 5). No differences between the various treatments in percentages of striped and dwarf plants were found. One family of three plants consisted of two plants with yellow/green stripes. These two plants set seed after open-pollination and the M_3 progenies of these plants gave *albina* and green seedlings. The percentages of *albina* seedlings for the two families were 19% and 30%. The green seedlings developed into both striped and completely green plants.

Leek plants on the trial field were visually examined for male sterility in 1992. The male sterility was confirmed by staining the pollen with aceto-carmine acid. Plants with less than 10% of stainable pollen were classified as male sterile. Pollen stainability of male sterile plants was very low (below 10%) as compared to male fertile plants of the control (more than 90%). A total of 111 male sterile plants (0.4%) were selected, with less than 10% of stainable pollen. Male sterile plants were found in the control as well. The NMU 10 mM-0.5h and 12.5 mM-0.5h treatments resulted in the highest percentages of male sterile plants (Table 5). The pooled percentage of male sterile plants for all treatments (0.5%) slightly exceeded that of the control (0.4%). M_2 progenies with only male sterile plants were not observed.

Table 5. Effects of different NMU treatments on the number and percentage of plants with yellow/green stripes, dwarf plants and male sterile plants in the M_2 of leek cv. Porino.

Mutagen	Conc. (mM)	Time (h)	Flowering plants Number	HS-families Number	Striped plants Number (%)	Dwarf plants Number (%)	MS-plants Number (%)
NMU	10 mM	0.5 h	10 547	542	11 (0.1) a[1]	0 (0) a[1]	50 (0.5) a[1]
NMU	10 mM	1.0 h	1 610	182	1 (0.1) a	16 (1.0) a	7 (0.4) a
NMU	12.5 mM	0.5 h	419	539	6 (0.1) a	7 (0.1) a	38 (0.6) a
NMU	12.5 mM	1.0 h	1 577	68	3 (0.2) a	0 (0) a	3 (0.2) a
control			3 051	133	0 (0) a	0 (0) a	13 (0.4) a

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

Nine M_2 progenies exhibited a relatively high percentage of male sterile plants, in particular the progenies derived from plant 204-07, 221-08 and 259-02 (Table 6). In order to examine the genetic background of the male sterility, all male sterile plants were isolated and open-pollinated by male fertile plants of different leek cultivars. The half-sib progenies subsequently were sown. Germination percentages of seeds from most progenies were high as compared to the control and the percentage of *albina* seedlings was comparable with that of the control (data not shown).

Table 6. Plant number, NMU treatment (1990), number of flowering plants per family and number and percentage of male sterile plants in the M_2 generation (1992) of leek cv. Porino.

Plant number M1	NMU treatment conc (mM)	time (h)	Flowering M_2 plants per family Number	MS M_2 plants per family Number	(%)
200-33	control		74	2	(3)
204-07	10 mM	0.5h	61	17	(28)
214-30	10 mM	0.5h	80	2	(3)
219-10	12.5mM	0.5h	77	3	(4)
221-08	12.5mM	0.5h	42	6	(14)
257-58	10 mM	0.5h	32	2	(6)
259-02	12.5mM	0.5h	14	2	(14)
259-54	12.5mM	0.5h	2	1	(50)
261-26	10 mM	0.5h	32	2	(6)

All the M_3 half-sib progenies flowered in 1993 and were examined for male sterility. Table 7 only shows the number and percentages of male sterile plants per M_3 half-sib family of the nine M_2 progenies. It is obvious from this table that the percentage of male sterile plants was very low in the M_3. Only the half-sib family 259-54 exhibited a relatively high number and percentage (40%) of male sterile plants. The other M_3 progenies of male sterile M_2 plants showed a similar low percentage of male sterile plants (data not shown).

Table 7. Number and percentage of male sterile plants after NMU treatment in M_3 progenies derived from male sterile M_2 plants of leek cv. Porino.

			M3 progenies 1993		
Plant number 1990 M1	NMU treatment conc time	MS-Plant number 1992	Flowering M_3 plants per family Number	MS M_3 plants per family Number	(%)
200-33	control	200-33-316	32	1	(3)
"	"	200-33-337	7	0	(0)
204-07	10 mM 0.5h	204-07-509	11	0	(0)
"	" "	204-07-510	9	0	(0)
"	" "	204-07-513	6	0	(0)
"	" "	204-07-514	18	0	(0)
"	" "	204-07-519	12	0	(0)
"	" "	204-07-521	11	0	(0)
"	" "	204-07-526	30	0	(0)
"	" "	204-07-527	24	0	(0)
"	" "	204-07-528	2	0	(0)
"	" "	204-07-529	21	0	(0)
"	" "	204-07-531	35	0	(0)
"	" "	204-07-534	5	0	(0)
"	" "	204-07-536	38	0	(0)
"	" "	204-07-537	24	1	(4)
"	" "	204-07-545	28	1	(4)
"	" "	204-07-614	25	0	(0)
"	" "	204-07-615	11	0	(0)
214-30	10 mM 0.5h	214-30-531	0	0	(-)
219-10	12.5mM 0.5h	219-10-315	19	1	(5)

Induction of (cytoplasmic) male sterility in leek by chemical mutagens

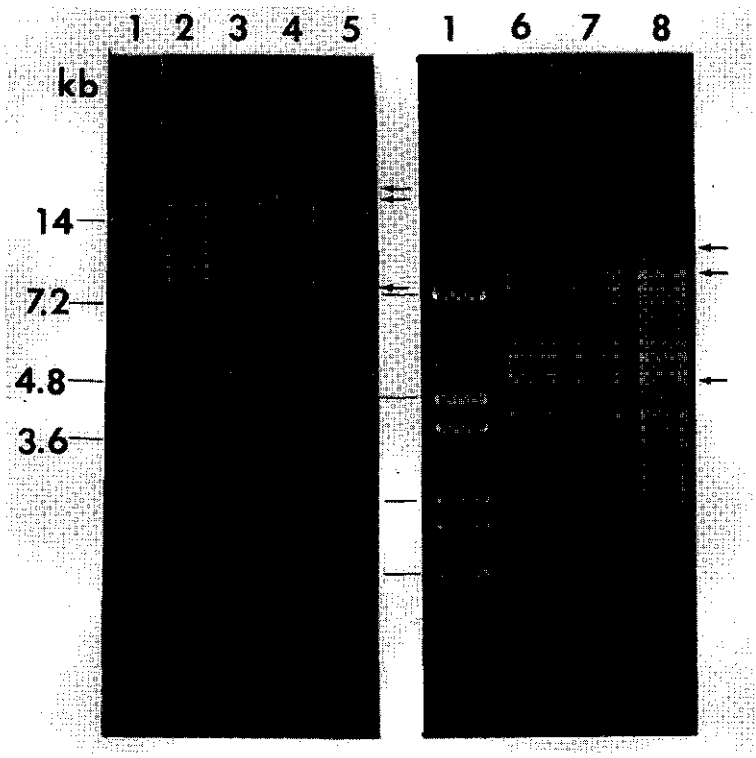

Fig. 3. *Bam*HI restriction patterns of mtDNAs from four male fertile plants of Porino (lanes 2-5) and three male sterile plants derived from the families 259-54-598, 204-07-513 and 221-08-642 (lanes 6, 7 and 8 resp.) of Porino. Lane 1: Lambda BstEII molecular weight marker. Arrows indicate differential fragments.

Discussion

Cytoplasmic male sterility (CMS) is one of a plant breeder's most desirable tools for producing hybrids, and is integrated in breeding programs of several crops. Despite considerable efforts during an extended period of time, it appears that the mutagenic treatments of leek seeds with NMU did not result in plants showing the desired CMS trait. Unsuccessful attempts to induce CMS also were reported for petunia after treatment with EB and N-nitroso-N-ethyl Urea (NEU) (Van Harten et al. 1985), sugar beet using acriflavine, EMS and STR (Brears and Lonsdale 1990) and leek using gamma-rays and NMU (Rauber and Grunewaldt 1991).

Several difficulties are met when inducing mutations in chloroplasts and mitochondria and in the recovery of the mutants. Nuclear genetic material is equally distributed to daughter cells during mitotic divisions, whereas mitochondria and plastids show a random distribution. Individual plastids and mitochondria are known to contain many copies of the same genome, and within each cell there are several organelles of each type. According to Bendich and Gauriloff (1984) actively dividing plant cells in meristems contain only a few mitochondria (7-8), organised as networks, while non-dividing cells may contain hundreds of mitochondria per cell. The time necessary for establishing a homoplasmon is solely dependent on the total number of mitochondria per cell and increases with increasing number of mitochondria per cell (Tilney-Bassett 1978). The formation of a homoplasmic cell for a mutant genome therefore requires segregation of the genomes within the organelle, and sorting out of the different organelle types. The fixation of a mutation in a mitochondrial genome population is probably further hampered by the frequent organelle fusion followed by DNA recombination and copy correction (Lonsdale et al. 1988).

Identification of a cytoplasmic mutation at the plant level requires also the formation of at least a mutant sector. Sorting out in the absence of selection pressure may take a long time, and in some cases the mutant phenotype is manifested only in the M_2, M_3 or later generations. There is some evidence that UV irradiation increases the rate of sorting-out of chloroplasts by lowering the effective copy number or by reducing the extent of cytoplasmic mixing (Sears and Sokalski 1991). Furthermore, the organization of embryo shoot meristems is of importance for all mutagenic treatments of seeds, since it determines whether a mutated cell may die or be lost during differentiation or may produce a cell progeny sufficient to be found throughout much of the plant including the germ cell line. On the average, it appears that only about five cells or less on the embryogenic shoot apex may become part of the germ line and thus contribute to the next generation (Brock 1979). A mutation induced after seed treatment with chemical mutagens will be produced in only one cell of the seed, and the mutated area is not likely to occur in the germ line. The expression of cytoplasmic male sterility mutations, furthermore, depends on the nuclear background, since fertility restorer genes can restore the male sterility trait and, therefore the search must be carried out in the appropriate lines.

In our experiments, most mutagens showed no effects on seed germination or percentage of chlorophyl deficiencies. NMU on the other hand turned out to be a very effective mutagen for leek seeds. The germination

percentage of leek decreased, and the number of *albina* seedlings increased with increasing NMU concentrations. This is in accordance with results of Tashiro et al. (1991) working on garlic mutants. The severe loss in number of surviving and flowering leek plants after treatment with *N*-nitroso-*N*-ethylurea (NEU) was also reported for leek by Van Harten et al. (1989) and, following NMU treatment of leek seeds, by Rauber and Grunewaldt (1991). Van Harten et al. (1985), after NEU treatment in petunia, also observed aberrant growth habits (e.g. bushy types, abnormal leaf types, dwarf types and chlorophyl aberrations) and an elevated percentage of chlorophyl deficiencies. Male sterility percentages in the M_1 generation were extremely high and varied between 9 and 33%, probably due to a high level of physiological damage. This is in accordance with results of Malinovskii et al. (1973) who obtained between 0.3% and 9.8% of male sterile sorghum plants after NMU treatments. Probably the high percentage is caused by the short post-washing time. According to Konzak et al. (1970) an increased post-washing time results in decreasing physiological damage but an unchanged chlorophyl mutation frequency. In unbuffered treatments of relatively long duration and at high temperatures significant physiological damage may result. The long duration treatments resulted in the highest percentage of male sterile plants. This is in accordance with results of Hagemann (1982), who recommended to use not too high concentrations of NMU, because there is a tendency for an increase in sterility with increasing concentrations of the mutagen used.

A remarkably high percentage (1%) of male sterile plants was found in the control. Van Harten et al. (1989) and Rauber and Grunewaldt (1991) also found a percentage of 1% of male sterile leek plants in the control, whereas Van Harten et al. (1989) only found between 0.7% and 1.6% of male sterile plants in the M_1. Rauber and Grunewaldt (1991) obtained between 2.3% and 4.2% of male sterile plants after a 10 mM NMU treatment for 0.5 h. Van Harten et al. (1989) and Rauber and Grunewaldt (1991) only examined the M_1 for induced male sterile plants. In the M_1, mutant sectors exhibiting male sterile flowers can be expected, but the large number of flowers per inflorescence makes it very difficult to detect these sectors. Selection in the M_2 and M_3 generation is generally recommended. We examined the M_2 generation, because the chances of recovering a CMS mutant in the M_1 generation are very low. The NMU treatments resulted in an elevated number of male sterile plants in the M_2, as compared to the control and the half-sib M_2 families 204-07, 221-08 and 259-02 produced the highest percentages of male sterile plants. However, it is important to take into consideration that

nuclear male sterility occurs within leek (Schweisguth 1970; Rauber 1989) and that genic male sterile plants can not be distinguished from cytoplasmic male sterile plants. It was therefore necessary to grow the M_3 generation in order to determine the genetic background of the male sterile plants. The M_3 progenies of these male sterile plants showed very low percentages of male sterility. A nuclear male sterility system is therefore supposed, because in the case of CMS high percentages (over 50%) were expected.

The possibility remained that the male sterility in the M_3 was suppressed by nuclear fertility restorer genes. This assumption was not supported by the observation that no additional variation in the mtDNA of male sterile plants was detected after restriction analysis. However, it was remarkable to find two mitochondrial types among control plants and male sterile plants. An additional study with more genotypes and with different leek cultivars resulted in identical results (Kik et al. in prep.). It is generally assumed that the variation in mtDNA and cpDNA between individuals or populations of the same plant taxa is negligible in comparison with the variation between taxa. However, in cultivated species, some studies have reported the existence of several mtDNA types within a species (Belhassen et al. 1993; Rouwendal et al. 1987).

Plastome mutations with chlorophyl deficiencies were found in the M_2 generation of leek, exhibiting striped white/green or yellow/green leaves, which is typical of the sorting-out of genetically different plastids (Tilney-Bassett 1975, 1978). The progenies segregated after open pollination into green, variegated, and white/yellow (*striata*) seedlings and plants. A cytoplasmic nature of the induced variegation is supposed, because of the high rates of *albina* and *striata* seedlings in progenies of *striata* plants.

Plastome mutants have been generated in numerous plant species by seed mutagenesis with NMU and NEU (Razoriteleva et al 1970; Travis et al. 1975; Hagemann 1976, 1982; Pohlheim 1981; Hosticka and Hanson 1984; Sharma and Sharma 1984; Fluhr et al. 1985; Börner and Sears 1986; Davidson et al. 1987; Walters et al. 1990; Martínez-Zapater et al. 1992). The most common mutations, those causing variegated or albino plants, are often maternally inherited, suggesting that they are carried by the chloroplasts. According to Hosticka and Hanson (1984) mitochondrial mutations can also result in abnormal growth and yellow/white striping. Newton and Coe (1986) proved that the affected organelle was the mitochondrion and not the chloroplast, despite the existence of "stripes". Therefore, it is not clear if this mutation is of chloroplast origin.

No source of CMS has been found, sofar, in leek or related forms of *A. ampeloprasum*. Although the search will continue, it is difficult to distinguish CMS from nuclear male sterility in the tetraploid leek. The high percentage of male sterile plants in the control, apparently, makes it profitable to look for spontaneously occurring male sterile plants in large leek seed production fields. There is a possibility that CMS is already present within leek but hidden due to the action of different restorer genes and two "sterilizing" cytoplasms. Therefore, it can be an attractive alternative to bring male sterile plants of both mitotypes into tissue culture and subsequently select for cytoplasmic revertants with a male fertile phenotype (cf. Kik et al. 1993). It is also possible that CMS from onion might be transferred to leek via protoplast fusion (Buiteveld, personal communication.). An alternative and at present more feasible approach is the utilization of genic male sterility by micropropagation (Pink 1993, Silvertand 1995).

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CHAPTER 4

The influence of physiological and environmental factors in flowering of leek (*Allium Ampeloprasum* L.)

B. Silvertand, R. Meijles, T. van der Made and A.M. van Harten

The Graduate School of Experimental Plant Sciences, Department of Plant Breeding, Wageningen Agricultural University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands

Summary. Early flowering of the biennial leek (*Allium ampeloprasum* L.) reduces the yield and quality of the product at harvest and is therefore a great disadvantage for growers. On the other hand, for leek breeders acceleration of flowering offers advantages, as it enables growing leek in a one-year cycle. In this study, the physiological and environmental factors which control inflorescence initiation and development were investigated. The duration of the juvenile phase was found to be strongly cultivar dependent. A prolonged vegetative stage or raising period (>10 weeks) resulted in increasing percentages of inflorescence primordia and flowering plants. A photoperiod of 16 h during the vegetative stage resulted in a high percentage of induced inflorescence primordia, but not in a higher percentage of plants which ultimately flowered. The percentage of inflorescence primordia significantly decreased with an increasing length of the vernalization period. A vernalization period of 6 weeks resulted in significantly the highest percentage of inflorescence primordia. The temperature, during the vernalization period, had no effect on the inflorescence development. Cultural methods and breeding efforts to prevent flowering in early leek varieties will not have much success.

Introduction

Leek is a mainly outbreeding autotetraploid ($2n=4x=32$) species, belonging to the diverse species complex *Allium ampeloprasum* L. (Van der Meer and Hanelt 1990). As a consequence every leek genotype is represented only by a single plant. Leek cultivars are, therefore, heterogeneous and unstable. Leek is widely grown in European countries, particulary in France, Belgium, The Netherlands and U.K., for its edible pseudostem formed by the leaf sheaths. Leek plants are biennials, i.e., they normally flower in the second season of their development. However, under specific conditions, they flower in the first season. The flower stalk of all *Alliums* is an apical extension of the stem, without histological differentiations into nodes and internodes (Rabinowitch 1990). Flowering of leek is of great economical importance during the two growing seasons. When grown for the pseudostem, the development of a flower stalk during the first season results in plants of a very poor quality. On the other hand, when grown for seeds, the seed yield directly depends on the amount of flowering.

The breeding process of leek is delayed because of the biennial nature of the crop. The development of a one-year growing cycle may be attractive for particular purposes; such as breeding for disease resistance, developing inbred

lines and performing backcrosses. However, for other breeding objectives, like selection for yield, winter hardiness, leaf colour and absence of bulbing, the one-year growing cycle is not suitable, since selection for these characters is performed in the second growing season (Van der Meer and Van Dam 1984).

Low temperatures and change from short day to long day conditions during the vegetative phase, are generally considered the most important causes for bolting in leek and those factors have been studied by several authors. David (1950) found that leek seeds could not be vernalized. Kazakova (1957) reported that short day-lengths (8 hours) led to later bolting and flowering, and lower seed production than obtained with longer day-lengths. Jones and Mann (1963) postulated that, like in the onion (*Allium cepa* L.), low temperatures during the winter period induce bolting in leek.

Additional studies under controlled conditions have established a number of relevant, but sometimes contradictory facts. Dragland (1972) found that leek plants did not require low temperatures to induce flower stalks. The day-length seemed to have little effect on the percentage of bolters. However, flower stalks were formed on plants with fewer leaves with increasing day-length. This work indicated that leek, with regard to bolting, can be characterised as a quantitative long day plant without a demand for vernalization, although it does react favourably to low temperatures. Daniël (1975) found that leek plants could be vernalized already 7-9 weeks after sowing. Temperature had no influence on flower induction and long day treatments, up to 13 h of light, elevated the number of flowering plants. A positive correlation between percentage of bolting plants and fresh weight of the seedlings at transplanting was found by Brewster and Steckel (1988). Plants raised at low temperatures (9°C) during vegetative growth gave a higher percentage of bolters than plants raised at higher temperatures and seedlings with just two visible true leaves were responsive. This indicates that low temperatures induced inflorescences. Plants raised in high nutrient conditions bolted more readily than plants of a similar fresh weight at the transplanting stage, raised in low nutrient conditions. Wiebe (1990) demonstrated that winter varieties of leek exhibit a juvenile period, whereas in summer and autumn varieties flower induction already may occur in the period just after sowing. Temperatures between 8 and 11°C were optimal for flower induction. Short day treatments during the vegetative growth period and long day treatments during the vernalization period enhanced subsequent flowering. Van der Meer and Van Dam (1984) realized a one-year growing cycle by vernalizing 10 weeks old plants during a period of 13 weeks at 10°C and long day conditi-

ons.

The sometimes contradictory results of the above mentioned experiments prompted us to a further study of the internal and external environmental factors which control inflorescence induction and development in leek. These factors can be of practical importance for both leek growers and breeders.

Materials and Methods

Plant material and growing conditions

Two experiments were performed from 1989 until 1991 with three Dutch leek cultivars: Alita (Royal Sluis, Enkhuizen), Strata (Royal Sluis, Enkhuizen) and Porino (Nunhems Zaden, Haelen). Alita is described as a fast-growing cultivar for early harvest in the summer period, Strata as an autumn variety and Porino as a winter leek.

In the first experiment, seeds of Porino were sown in plastic boxes (100 seeds per box; 16 h photoperiod; Son/T Agro 27 $W.m^{-2}$), and raised for either 6, 10, 12, 14 or 16 weeks in a greenhouse at 21°C. After this period the boxes were transferred to climate chambers for vernalization treatments of 4°, 6°, 9°, 13°, 18° or 21°C and 8, 12 or 16 h photoperiod (16 $W.m^{-2}$) per day. The duration of this treatment was either 6 or 12 weeks. Subsequently the plants were transplanted into pots (20 cm ϕ) and placed in a greenhouse (21°C) with a photoperiod extended to 16 h (Son/T Agro 27 $W.m^{-2}$). Untreated control plants were raised in the greenhouse at 21°C and 16 h photoperiod (Son/T Agro 27 $W.m^{-2}$) in pots (20 cm ϕ). Each treatment consisted of 50 plants.

In the second experiment, seeds of Alita, Strata and Porino were sown in boxes (100 seeds per box) and grown at 21°C. The photoperiod was either 8 or 16 h (Son/T Agro 27 $W.m^{-2}$) and the raising period lasted 6, 10 or 14 weeks. After this period the boxes were transferred to climate chambers for vernalization at either 9° or 13°C and 8 or 16 h photoperiod (16 $W.m^{-2}$) per day. The duration of this vernalization treatment was 6, 9 or 12 weeks. The plants were subsequently transplanted to the soil in two replicates per treatment of 50 plants each in a polyethylene tunnel. Control plant were raised in boxes (100 seeds per box) in a greenhouse at 21°C, with either 8 or 16 h photoperiod (Son/T Agro 27 $W.m^{-2}$). The experiment was set up in a chronological order, to make sure that plants from all treatments could be retransplanted to the soil at the same moment.

Experiments and Statistical analysis

In the first experiment, the number and percentage of flowering plants were recorded per treatment, five months after the transfer to the greenhouse. In the second experiment, the average length of the longest leaf, the number of leaves, the number of roots, the fresh-weight and the dry-weight for ten plants per treatment was determined, both at the start and at the end of the vernalization treatment. The number of leaves was counted when the flower stalks became visible above the leaf sheaths and, subsequently, the plants were removed from the field plots. The experiment was terminated after a period of five months. The remaining plants were dissected to check for concealed inflorescences. The sizes of these young inflorescences varied from a few mm to several cm and were termed primordia. The percentages of plants with inflorescence primordia and flowering plants were recorded as well as the total time required from sowing until the appearance of the flower stalk. A One-Way analysis of variance (F-test) was used to check for treatment effects. Subsequently, significant treatment differences were analysed by calculating Student's '*t*' with $P \leq 0.05$.

Results

First experiment

In this experiment, the effects of the plant age, vernalization temperature, day-length during vernalization and the duration of the vernalization-period on the number and percentage of flowering leek plants were studied. It is obvious from all treatments that the number of flowering plants was very low and, therefore, interpretation of the results must be performed with extreme precaution (Table 1).

The age of the plants before the start of the vernalization period affected the number of flowering plants. A raising period of 10 weeks resulted in the highest percentage (18%) of flowering plants. Optimum vernalization temperatures from 6° to 9°C were found, whereas a temperature of 21°C resulted in the lowest number of flowering plants. The number of flowering plants also increased with increasing day-length and vernalization period. A day-length of 16 h and a vernalization period of 12 weeks resulted in the highest percentages of flowering plants.

Table 1. Effects of plant age before vernalization, temperature and day-length during vernalization and duration of vernalization period on number and percentage of flowering leek plants, five months after vernalization.

Treat-ment	Plant Number	Flowering plants (%)
Age (weeks)		
6	50	4
10	50	18
12	50	8
14	50	12
16	50	8
Temp (°C)		
4	50	6
6	50	14
9	50	10
13	50	8
18	50	4
21	50	2
Day-length (h)		
8	50	6
12	50	6
16	50	10
Duration (weeks)		
6	50	4
12	50	10
control	50	4

Second experiment

The results of the One-Way analysis of variance are summarized in Table 2 and only the results of the significant ('*t*'-test) treatment effects will be discussed and some displayed (Table 3). The cultivar choice significantly determined the number of leaves both at the end of the vernalization period

and the flowering period, as well as the time before the appearance of the flower stalks. Alma significantly had the lowest number of leaves both shortly after vernalization, and at the moment of appearance of the flower stalks. This cultivar also showed the shortest period (219 days) between sowing and the appearance of the flower stalks (Table 3).

The duration of the raising period before vernalization and the day-length during this period significantly influenced both the plant development during this period and during the following vernalization period. Significant effects of both treatments on leaf-length, number of leaves, fresh-weight and dry-weight at the end of the raising period, and leaf-length, number of leaves, fresh-weight and dry-weight at the end of the vernalization period were found. The mean values for all parameters significantly increased with increasing duration of the raising period and increasing day-length during this period (data not shown). The duration of the raising period also significantly influenced the percentage of inflorescence primordia, the percentage of flowering plants and the time before appearance of the flower stalks (Table 3). Percentages of plants with both inflorescence primordia and flowering plants were significantly higher after a raising period of 10 weeks as compared to 6 weeks. These percentages further increased with an increasing raising period. A longer raising period resulted in a later appearance of the flower stalks. The day-length during the raising period significantly influenced the percentage of inflorescence primordia. A photoperiod of 16 h during this period resulted in a significantly higher percentage of induced inflorescence primordia than that of 8 h, but not in a higher percentage of flowering plants.

The duration of the vernalization period significantly influenced the further development of the plants during this period (leaf-length, number of leaves, fresh-weight and dry-weight), the number of leaves at appearance of the flower stalk, and the percentage of inflorescence primordia. Extended vernalization periods resulted in significantly higher mean values for leaf length, leaf number, fresh- and dry-weight, but in a lower number of leaves at the moment of flowering (data not shown). The percentage of inflorescence primordia significantly decreased with an increasing vernalization period (Table 3). A period of 6 weeks resulted in significantly the highest percentage of inflorescence primordia. The day-length during the vernalization period only influenced the leaf-length. A 16 h day-length, in this respect, resulted in significantly the longest leaves. The temperature during vernalization significantly influenced the number of leaves and fresh-weights at the end of the vernalization period. A 13°C treatment resulted in

Table 2. Results from One-Way analysis of variance between all pairs of treatments and independent parameters of leek. A significant (F test, $P \leq 0.05$) effect is represented by a "+".

Treatment	Parameters											
	LLRA[1]	NLRA[2]	FWRA[3]	DWRA[4]	LLVE[5]	NLVE[6]	FWVE[7]	DWVE[8]	NLFL[9]	PPFL[10]	PFFL[11]	TIFL[12]
CULT[13]	-[19]	-	-	-	-	+	-	-	+	-	-	+
DURA[14]	+	+	+	+	+	+	+	+	-	+	+	+
DARA[15]	+	+	+	+	+	+	+	+	-	+	-	-
DUVE[16]					+	+	+	+	+	+	-	-
DAVE[17]					+	-	-	-	-	-	-	-
TEVE[18]					-	+	+	-	-	-	-	-

[1] LLRA = Leaf-Length at the end of the Raising period,
[2] NLRA = Number of Leaves at the end of the Raising period,
[3] FWRA = Fresh-Weight at the end of the Raising period,
[4] DWRA = Dry-Weight at the end of the Raising period,
[5] LLVE = Leaf-Length at the end of the Vernalization period,
[6] NLVE = Number of Leaves at the end of the Vernalization period,
[7] FWVE = Fresh-Weight at the end of the Vernalization period,
[8] DWVE = Dry-Weight at the end of the Vernalization period,
[9] NLFL = Number of Leaves at the end of the Flowering period
[10] PPFL = Percentage of plants with inflorescence Primordia observed in the Flowering period,
[11] TFFL = Percentage of Flowering plants in the Flowering period,
[12] TIFL = Time required before appearance of Flower stalks,
[13] CULT = CULTivars,
[14] DURA = Duration of the Raising period,
[15] DARA = Day-length during the Raising period,
[16] DUVE = Duration of the Vernalization period,
[17] DAVE = Day-length during the Vernalization period,
[18] TEVE = Temperature during the Vernalization period,
[19] - = no significant effect,
+ = significant effect

Table 3. Significant effects of cultivar choice (CULT), duration of the raising period (DURA), day-length during the raising period (DARA) and the duration of the vernalization period (DUVE) on the percentage of plants with inflorescence primordia (PPFL), percentage of flowering plants (PFFL) and on the time required before the appearance of flower stalks (TIFL) of leek.

Treatment	PPFL (%)	PFFL (%)	TIFL (days)
CULT			
Alma			219 a[1]
Strata			234 b
Porino			230 b
DURA (weeks)			
6	17.1 a	13.2 a	201 a
10	27.0 b	23.6 b	219 b
14	29.0 b	26.6 b	255 c
DARA (h)			
8	23.5 a		
16	28.2 b		
DUVE (weeks)			
6	28.5 b		
9	23.0 a		
12	21.6 a		

[1] Treatments with different letters are significantly different at the 5% significance level ('t'test)

the highest mean values for number of leaves and fresh-weights.

The time necessary for the development of the inflorescence from inflorescence primordia depended on the cultivar and the duration of the raising period (Table 3). Alita flowered 2 weeks earlier than Strata and Porino. It seems that this period is a constant for each cultivar under specific environmental circumstances.

Significant interactions were found between the duration of the raising period and the duration of the vernalization period on the percentage of induced inflorescence primordia, percentage of flowering plants and the time before flower stalks appeared. A 14 weeks raising period followed by a 6 weeks vernalization period gave on average the same results as a 10 weeks

raising period, followed by a short vernalization period resulted in a relatively high percentage of flowering plants. In general, the longer the raising period the higher the percentage of eventually flowering plants.

Commercial leek growers in the Netherlands who aim at an early harvest starting in June, use fast growing cultivars and grow the plants at low temperatures (12°-15°C) and extended day-lengths to support the vegetative growth and to ensure that a well-developed and hardened plant (± 12 weeks) can be transplanted to the field, already, in April. During the raising period these plants reach the appropriate physiological age to become sensitive to flower inducing circumstances. Both low temperatures during the raising period and, natural low temperatures after transplanting to the field in April and May, may result in the development of inflorescence primordia and the appearance of flower stalks. Leek cultivars grown for an early harvest are therefore "doomed" to flower. Environmental conditions during the following months, genotypic differences between plants and the time of harvest will further determine the percentage of eventually flowering plants. The percentage of flowering plants at the time of harvest can be reduced only by reducing the length of the raising period. However, by reducing the length of this period or by reducing the day-length, the seedling weights will be reduced as well, and, according to Brewster and Steckel (1988), there is a positive correlation between the yields of leek at harvest and the fresh-weight of seedlings at transplanting.

In conclusion, it seems not so easy to select for bolting-resistance because selection for this trait results in lower growth rates and, consequently, later harvest or a reduced yield.

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CHAPTER 5

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

B. Silvertand, A.M. van Harten, J. Mazereeuw, P. Lavrijsen and E. Jacobsen

The Graduate School of Experimental Plant Sciences, Department of Plant Breeding, Wageningen Agricultural University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands

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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 power-ful tool in hybrid leek breeding.

Introduction

Leek (*Allium ampeloprasum* L.) is an autotetraploid (2n=4*x*=32) mainly out-breeding 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.

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% ethanol and 20 min in 1.5% 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 and the average number of regenerated shoots per explant was calculated and recorded. 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 and the average number of regenerated shoots 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 percentage of regenerating explants and average number of regenerated shoots were found between the 29 plants (Table 1). The percentage of regenerating explants varied between 10 and 100 percent. An overall percentage 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 average number of regenerated

Table 1. Percentage of regenerating explants (PREX) and average number of regenerated shoots per explant (NSHO) for 29 genotypically different plants of leek cv. 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)

shoots 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 percentage of regenerating explants and average number of regenerated shoots. For example, the percentage of regenerating explants and average number of regenerated shoots 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 percentage of regenerating explants (resp. -0.13 and -0.46) and between flower stalk length and average number of regenerated shoots (resp. -0.13 and -0.21). A relatively long flower stalk resulted in a reduced percentage of regenerating explants and average number of regenerated shoots for all plants cultured.

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 and average number of regenerated shoots per explant 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 percentage of regenerating explants and average number of regenerated shoots, whereas a combination of 10 $mg.l^{-1}$ BAP and 10 $mg.l^{-1}$ NAA resulted in both the highest percentage of regenerating explants and average number of regenerated shoots. Similar to the experiments with cultivar Vrizo, differences were found in percentage of regenerating explants and average number of regenerated shoots between the two genotypes. Por-286 showed the highest total percentage of regenerating explants (84%) and total average number of regenerated shoots (26). The percentage of regenerating explants and average number of regenerated shoots for both genotypes were not influenced significantly, in this experiment, by the flower stalk length. A decrease in percentage of

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 leek cv. 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

[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).

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 leek cv. 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)

regenerating explants 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 percentage of regenerating explants and average number of regenerated shoots decreased with increased flower stalk length and that a flower stalk length of 10 cm resulted in an optimal average number of shoots. For Por-286 we noticed, however, that a flower stalk length of 10 cm resulted in the lowest percentage of regenerating explants (67%).

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 were found between basal and apical explants (Table 3). However, significant differences between the four genotypes in percentage of regenerating explants were observed: Por-1 showing the lowest and Por-4 the highest percentage. In general, basal explants produced, in comparison with apical ones, significantly the highest average number of shoots per explant (Table 3). By contrast, apical explants from Por-2 showed a higher average number of shoots than basal explants. Significant differences between the four genotypes in average number of shoots were found. Por-1 gave the lowest and Por-4 the highest average number.

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 leek cv. 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.

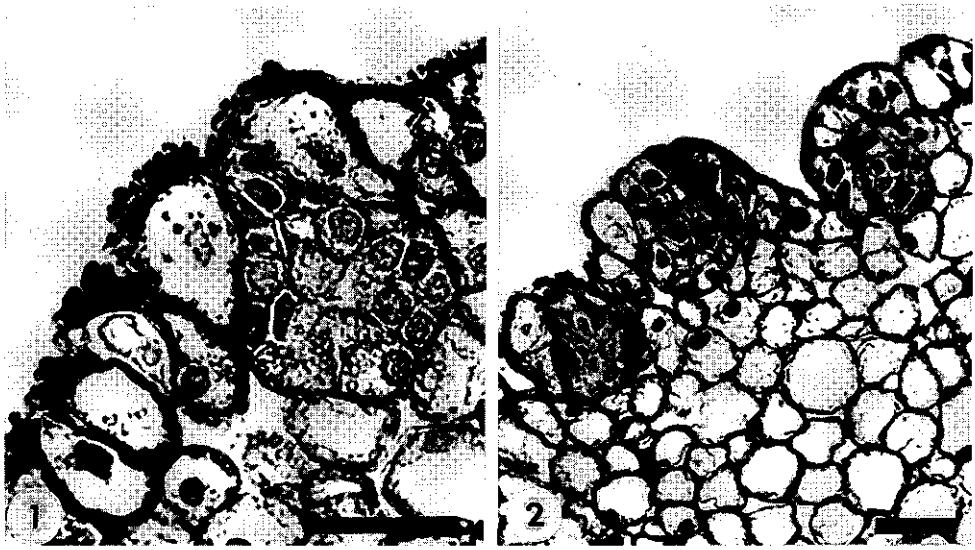

Fig.1. Transverse section near the basal cut end of a leek flower stalk explant, showing active cell division within single subepidermal cells (Bar= 5μm); **Fig.2.** Transverse section showing young shoot primordia emerging through epidermis of leek flower stalk explant (Bar=5μm).

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.

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 the percentage of regenerating explants and the number of regenerated shoots 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 the percentage of regenerating explants and the number of regenerated shoots. 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 percentage of regenerating explants and the number of regenerated shoots 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 percentage of regenerating explants and number of regenerated shoots.

The length of the flower stalk and the origin of the explant influenced the percentage of regenerating explants and number of regenerated shoots.

The length of the flower stalk is a measure of the physiological age. Relatively long and old flower stalks showed a reduced percentage of regenerating explants and number of regenerated shoots. Basal flower stalk explants showed a higher number of shoots 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 percentage of regenerating explants and number of regenerated shoots 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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CHAPTER 6

Plant regeneration via organogenesis and somatic embryogenesis in callus cultures derived from mature zygotic embryos of leek (*Allium ampeloprasum* L.)

B. Silvertand, A. van Rooyen, P. Lavrijsen, A.M. van Harten and E. Jacobsen

The Graduate School of Experimental Plant Sciences, Department of Plant Breeding, Wageningen Agricultural University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands

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Summary. A high frequency plant regeneration system via organogenesis and somatic embryogenesis was established with callus cultures derived from mature zygotic embryos of different leek genotypes (*Allium ampeloprasum* L.). Four different callus types with varying morphogenetic potential were obtained. Relatively high concentrations of the auxin 2,4-dichlorophenoxy-acetic acid reduced callus weight and subsequent shoot regeneration and primordia formation of the callus. Shoot regeneration and primordia formation of the callus decreased after prolonged subculture on media containing 2,4-dichlorophenoxy acetic acid. A callus growth period of six weeks on Murashige and Skoog medium with 0.25-0.5 $mg.l^{-1}$ 2,4-dichlorophenoxy acetic acid showed the highest rate of shoot regeneration after transfer of callus to regeneration medium with 1 $mg.l^{-1}$ kinetin.

Differences between leek genotypes in callus type, callus weight, shoot regeneration and primordia formation were observed. Histological observations showed that plant regeneration took place, both via the pathway of somatic embryogenenesis and organogenesis.

Introduction

Leek (*Allium ampeloprasum* L.) is an autotetraploid (2n=4*x*=32), mainly outbreeding and monocotyledonous crop, grown for its edible (false) stem.

The lack of efficient regeneration systems for many monocotyledon species has slowed down the progress of their genetic modification. In the Netherlands, much effort is being undertaken for developing *in vitro* techniques such as somatic hybridization, genetic transformation and *in vitro* selection for mutants, in order to support the breeding programmes of leek. For application of these techniques, an efficient callus regeneration system as well as selection of good regenerating genotypes is required.

Plant regeneration *in vitro* occurs via organogenesis and/or somatic embryogenesis. Organogenesis is characterized by the production of unipolar bud primordium with subsequent development into a shoot. The shoot then becomes rooted via root organogenesis (Brown & Thorpe 1984). The shoots and roots are either directly formed upon the explant or indirectly on callus tissue. Somatic embryogenesis is the development of somatic cells through an orderly series of characteristic morphological stages into structures that resemble zygotic embryos. Somatic embryos may form directly on the explant or indirectly from callus aggregates or suspension cells (Emons 1994). *In vitro* shoot regeneration via direct and indirect organogenesis, using different explants of the donor plant, has been described for several *Allium*

species (for reviews, see Novák et al. 1986a; Novák et al. 1986b and Novák 1990).

Stem explants, basal plates, leaf discs, flower heads, open flowers and single flower bud receptacles have been used as explant material for *in vitro* multiplication of leek (Debergh & Standaert-De Metsenaere 1976; Dunstan & Short 1979; Novák & Havel 1981; Van Doorne et al. 1988; Rauber & Grunewaldt 1988; Doré 1988; Baumunk-Wende 1989).

Several authors reported plant regeneration of *Allium* as somatic embryogenesis. Somatic embryos occur in cultures of seedling radicle tissue, seedling shoot meristem tissue, basal plate tissue and explants of flower heads (Abo El-Nil 1977; Dunstan & Short 1978; Phillips & Luteyn 1983; Shahin & Kaneko 1986; Havel & Novák 1988; Lu et al. 1989; Keller 1992; Van der Valk et al. 1992). Somatic embryogenesis in leek (*A. ampeloprasum* L.) has only recently been reported by Van der Valk et al. (1992) and Buiteveld et al. (1993).

The plant regeneration efficiency in monocotyledonous species is influenced strongly by factors such as explant type, culture conditions and genotype (Bhaskaran & Smith 1990; Novák 1990). In this study, the aim was to characterize and optimize medium and culture factors leading to a reproducible and highly efficient in vitro callus culture and callus regeneration system for various leek genotypes.

Materials and methods

Plant material. In a first experiment, seeds from several commercial leek cultivars were used (Castelstar, Gavia and Snowstar from Enza Zaden, Enkhuizen; Porino and Farinto from Nunhems Zaden, Haelen; Pancho from Nickerson-Zwaan, Barendrecht; Dakota, Kilima and Strata from Royal Sluis, Enkhuizen).

In order to study medium and culture factors, we produced genetically more uniform explant material. Seeds were harvested from selfings and crosses between selected genotypes. These seeds showed a relatively high *in vitro* shoot regeneration, as compared to explant material from the original cultivars. The numbers V14, V17, V22 and V43 represent seeds from individual full-sib families, derived from crosses between such selected genotypes and were used in the other experiments. Full-sib family V14 originated from a cross between one genotype from cultivar Castelstar and one from Farinto, V17 from a cross between one genotype from Dakota and one from Farinto, V22 from a cross between two Castelstar genotypes and

V43 from a cross between two Dakota genotypes.

Explants. Seeds were surface sterilized for 30 sec in 70% ethanol and 20 min in 1% sodium hypochlorite (commercial bleach) with a drop of Tween 20, and subsequently rinsed several times (5, 10, 15 and 20 min) in sterile distilled water. Seeds were then stored in a refrigerator at 5°C over night. After this period of imbibition, seeds were sterilized for a second time. After sterilization, the mature zygotic embryo, consisting of the short primary root, shoot apex and a small part of the long curved cotyledon, was isolated from the seed.

Media and Culture Conditions. For callus induction and growth, explants were placed on callus induction medium, consisting of MS salts and vitamins (Murashige and Skoog 1962), 30 $g.l^{-1}$ sucrose and 8 $g.l^{-1}$ agar (Daishin, Brunschwig). Different concentrations of 2,4-D ranging from 0.25 $mg.l^{-1}$ to 8 $mg.l^{-1}$ were used in the experiments. Media were adjusted to pH 5.8 prior to autoclaving at 1.1 bar, 120°C for 20 min. Cultures in Petri dishes (4 cm diameter) were incubated in the dark at 21 °C and calluses were subcultured at three week intervals on the same medium.

For regeneration, calluses (8 weeks old) were transferred to MS medium, supplemented with 30 $g.l^{-1}$ sucrose, 8 $g.l^{-1}$ agar and 1 $mg.l^{-1}$ kinetin and cultured in Petri dishes (4 cm diameter). Cultures were placed in the light, approx. 20 $W.m^{-2}$, (16/8 h photoperiod) at 21°C and subcultured at three week intervals on the same medium. After the first subculture, calluses were transferred to glass jars. After 8 weeks, regenerating calluses were cultured on half-strength MS medium, 20 $g.l^{-1}$ sucrose and 8 $g.l^{-1}$ agar for further development of shoots and roots. Plantlets were separated individually and transferred to soil, after another 4-6 weeks, where they were kept at a high humidity for 1-2 weeks. Transferring to soil was rather successful, although smaller plants (e.g.< 3 cm) often died.

Experiments and Statistical analysis. At the end of the callus initiation and growth period, callus type and total callus weight were recorded per excised embryo. After the regeneration period, shoots measuring more than 5 mm were counted and classified. At the same time, several green shoot primordia were also observed. They were classified as shoot primordia and counted. No distinction was made between plantlets obtained via organogenesis or somatic embryogenesis. All of them were classified as shoots. Callus pieces were not split during the callus growth and regeneration period. Data are related to one

excised embryo. Distribution of the data was not normal in our experiments. Results were therefore analysed using the Mann-Whitney 'U'-test.

Histology. For histological studies, tissues were fixed in 5% 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 & O'Brian 1968) and examined with a light microscope.

Results

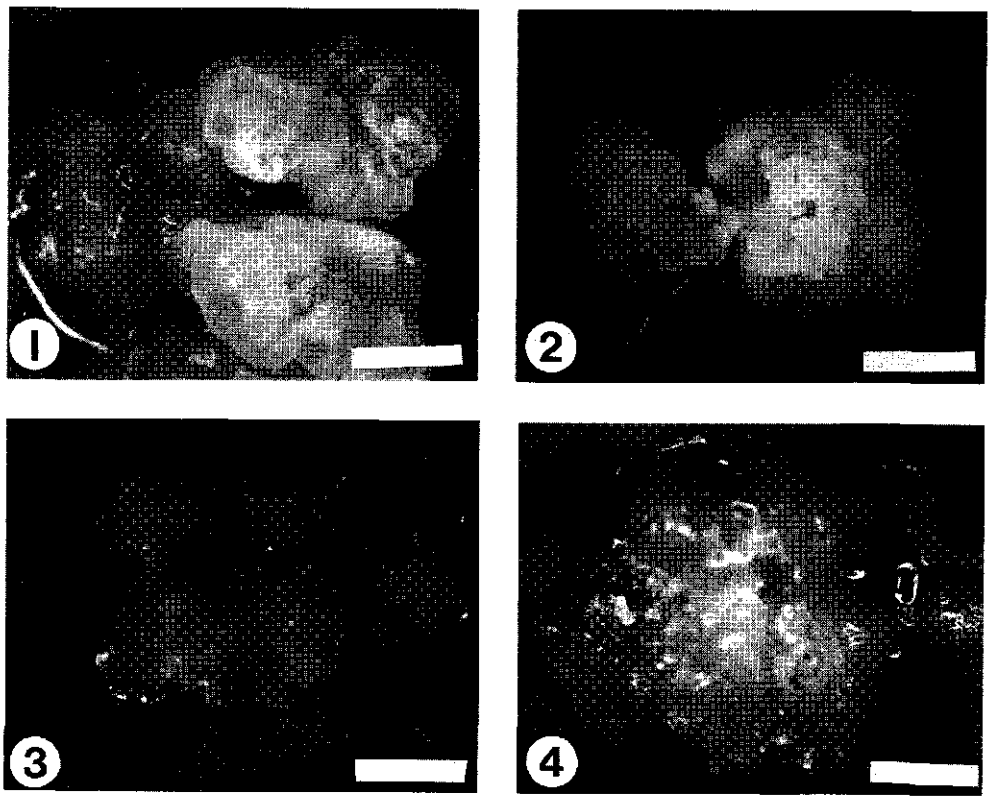
Callus types.

Mature zygotic embryos were isolated from 2325 genotypically different seeds belonging to the leek cultivars Castelstar, Gavia, Snowstar, Porino, Farinto, Pancho, Dakota, Kilima and Strata. After 3 weeks on callus induction medium, containing 1 $mg.l^{-1}$ 2,4-D, 635 embryos (27%) showed no response and were no longer cultured. The explants of the remaining 1690 embryos were subcultured and produced different callus types.

Four types of callus (Figs. 1-4), with varying morphogenetic potential, were obtained (Table 1): (I) Compact callus (54%): A white, compact, hard and highly organized callus tissue with lobate, fan-shaped structures. Sometimes localized areas of white and embryogenic callus and many globular or embryo-like structures appeared on the surface of this callus tissue; (II) Globular callus (7%); (III) Soft callus (10%): A yellowish-brown loose, crystalline unorganised callus; (IV) Gelatinous callus (29%): A yellowish-brown watery callus producing a gelatinous substance on its surface.

The compact callus type was most frequently observed. Total callus weight and the number of regenerated shoots and shoot primordia per excised embryo, were determined for the four callus types.

Callus weight of type II was significantly higher than callus weight of type I and IV. No significant differences in callus weight between type I and III and type II and III were found. Type IV exhibited the lowest callus weight. A total average of 0.05 g callus was produced, per excised and callusing embryo, after 8 weeks of culture by all genotypes.



Figs. 1-4. Four different leek callus types after 8 weeks on callus induction medium with 1 $mg.l^{-1}$ 2,4-D.; 1 Compact callus; 2 globular callus; 3 crystalline callus; 4 gelatinous callus (Bar = 2.5 mm).

Callus type III and IV produced the lowest number of shoots and shoot primordia and type I and II the highest. Type II callus produced significantly more shoots and shoot primordia than all the other types (14 and 26 respectively). An average of 7 shoots and 11 shoot primordia per excised embryo was found for all the genotypes tested. Genotypes capable of producing more than 100 shoots were found as well (data not shown).

Table 1. Average callus weight, number of shoots and shoot primordia per excised embryo for different callus types derived from 1690 leek plants with different genotypes.

Callus type[1]	N[2]	(%)	Callus weight (in g)[3]	Shoot primordia Number[3]	Shoots Number [3]
I	914	(54)	0.05 a[4]	14 a	9 a
II	120	(7)	0.06 b	26 b	14 b
III	164	(10)	0.06 ab	4 c	2 c
IV	492	(29)	0.03 c	3 c	2 c
TAV[5]	1690	(100)	0.05	11	7

[1] I = Compact callus, II = Globular callus, III = Soft callus, IV= Gelatinous callus
[2] N = number of cultured callus pieces classified per callus type, (%)= percentage
[3] Callus weight per excised embryo after 8 weeks of culture on callus growth medium (MS+ 1 mg.l^{-1} 2,4-D). Number of shoots and shoot primordia per excised embryo after 9 weeks on regeneration medium (MS+ 1 mg.l^{-1} kinetin)
[4] Within each column treatments with different letters are significantly different at the 5% level (Mann-Whitney test)
[5] Total average

Effect of 2,4-D concentration on callus type, callus weight, shoot regeneration and primordia formation.

Mature zygotic embryos were isolated from seeds of full-sib V17. Embryos (60 per treatment) were placed on media with varying 2,4-D concentrations (0, 0.25, 0.5, 1, 2 or 8 mg.l^{-1}).

Without 2,4-D in the medium, germination of the embryo occurred. Only 12% of the excised embryos showed no callus development after 3 weeks of callus culture. Compact callus was mainly produced at lower 2,4-D concentrations (0.25, 0.5 and 1 mg.l^{-1} 2,4-D), whereas higher concentrations (2 and 8 mg.l^{-1}) gave more soft and watery callus (Table 2). High 2,4-D concentrations also resulted in more root formation. Globular callus was observed mainly in the 0.25 mg l^{-1} treatment. At low 2,4-D concentrations significantly more callus was produced and significantly more shoots and shoot primordia were obtained after transfer of the callus to regeneration medium. A concentration of 0.25 mg.l^{-1} 2,4-D resulted in the highest number of shoots, although this number of shoots did not differ significantly from the number of shoots of the 0.5 mg.l^{-1} treatment.

Table 2. Effect of different 2,4-D concentrations on callus type, average callus weight, number of shoots and shoot primordia. Explant material derived from full-sib V17 of leek.

2,4-D (in $mg.l^{-1}$)	N[1]	Callus type (%)			Callus weight (in g)[3]	Shoot primordia Number[3]	Shoots Number[3]
		I	II	(III+IV)[2]			
0.25	53	66	17	17	0.06 a[4]	51 a	9 a
0.5	53	74	6	20	0.05 a	63 a	8 a
1.0	45	76	4	20	0.03 b	47 a	5 b
2.0	47	50	4	46	0.02 c	26 b	3 b
8.0	53	46	0	54	0.01 d	8 c	1 c

[1] Number of callus pieces obtained from 60 excised embryos

[2] I = Compact callus, II = Globular callus, III = Soft callus, IV= Gelatinous callus. Callus type III and IV pooled

[3] Callus weight per excised embryo after 8 weeks of culture on callus growth medium (MS+ 2,4-D). No. of shoots and shoot primordia per excised embryo after 9 weeks on regeneration medium (MS+ 1 $mg.l^{-1}$ kinetin)

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

Effect of duration of callus growth on callus type, callus weight, shoot regeneration and primordia formation.

In preliminary experiments, we noticed that the regeneration potential declined when leek callus had been cultured for a prolonged period (>12 weeks) on medium with 1 $mg.l^{-1}$ 2,4-D. After 14 weeks, most of the calluses lost their shoot forming ability, became brown and were only capable of producing roots; although some specific genotypes remained regenerative for more than one year.

We studied the effect of the duration of the callus growth on callus type, callus weight and successive shoot regeneration. The duration of the callus growth ranged from 4 to 12 weeks and the explant material came from the full-sibs V14 and V43. Per full-sib family, 30 explants were cultured. An average of 11% of the excised embryos of V14 and 4% of V43 showed no callus development after 3 weeks of callus culture. The percentage of compact and globular callus increased after a prolonged callus culture at the cost of the percentage of watery and soft callus (Tables 3a and 3b). Most globular callus was observed after 10 and 12 weeks.

It was obvious that callus weight increased after prolonged callus culture. The largest increase in callus weight occurred mainly between week

8 and 10.

The number of regenerated shoots of full-sib V14 decreased significantly after a prolonged callus growth period. Only one shoot per callus piece was obtained after 12 weeks, despite the relative increase in proportion of compact and nodular callus types. A callus growth period of 4 weeks was too short for optimal shoot production. Shoot regeneration was

Table 3a. Effect of duration of callus maintenance period on callus type, average callus weight, number of shoots and shoot primordia. Explant material derived from seeds of full-sib V14 of leek.

Duration in weeks[1]	N[2]	Callus type (%) I	II	(III+IV)[3]	Callus weight (in g)[4]	Shoot primordia[4] Number	Shoots Number[4]
4	29	79	0	21	0.00 a[5]	15 a	2 ab
6	26	73	0	27	0.01 b	15 a	4 a
8	29	69	1	30	0.01 b	31 a	2 ab
10	23	87	3	13	0.03 c	30 a	2 ab
12	26	81	11	8	0.04 c	34 a	1 b

Table 3b. Effect of duration of callus maintenance period on callus type, average callus weight, number of shoots and shoot primordia. Explant material derived from seeds of full-sib V43 of leek.

Duration in weeks[(1)]	N[2]	Callus type (%) I	II	(III+IV)[3]	Callus weight (in g)[4]	Shoot primordia Number[4]	Shoots Number[4]
4	30	70	13	17	0.01 a[5]	31 a	5 ab
6	27	78	4	18	0.02 b	56 a	6 a
8	30	74	3	23	0.04 b	44 a	4 ab
10	27	66	30	4	0.10 c	43 a	2 bc
12	30	90	10	0	0.15 c	28 b	1 c

[1] Duration of the callus maintenance period in weeks

[2] Number of callus pieces obtained from 30 excised embryos

[3] I = Compact callus, II = Globular callus, III = Soft callus, IV= Gelatinous callus. Callus type III and IV pooled.

[4] Callus weight per excised embryo at the end of the callus maintenance period on callus growth medium (MS+ 1 mg.l^{-1} 2,4-D). No. of shoots and shoot primordia per excised embryo after 9 weeks on regeneration medium (MS+ 1 mg.l^{-1} kinetin)

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

maximal for both full-sibs, when callus was cultured for 6 weeks on callus growth medium.

There was a clear difference in callus weight and shoot regeneration between both full-sibs. Full-sib V43 showed a higher callus weight, shoot regeneration and primordia production than V14.

Effect of a short growth period with relatively high 2,4-D concentrations on callus type, callus weight, shoot regeneration and primordia formation.

Embryos (60 per treatment) from genotypes of full-sib V22 were placed on a medium with 4 or 8 mg.l^{-1} 2,4-D for 1, 2 or 4 weeks (Table 4). After this period, the calluses were transplanted to media only containing 0.25 mg.l^{-1} 2,4-D. The control consisted of explants cultured for 8 weeks on medium with 0.25 mg.l^{-1} 2,4-D. After 8 weeks, calluses of all treatments were transferred to regeneration medium.

Table 4. Effect of a short growth period with relatively high 2,4-D concentrations on average callus weight, number of shoots and shoot primordia from full-sib V22 of leek.

Treatment[1]	N[2]	Callus type (%) I	II	(III+IV)[3]	Callus weight (in g)[4]	Shoot primordia Number[4]	Shoots Number[4]
1 - 4.0	54	81	6	13	0.02 ab[5]	25 a	2 ab
1 - 8.0	51	80	2	18	0.02 a	25 a	3 ab
2 - 4.0	51	71	4	25	0.01 bc	20 a	2 ab
2 - 8.0	47	68	9	23	0.01 bc	19 a	4 a
4 - 4.0	50	66	0	34	0.01 bc	20 a	3 ab
4 - 8.0	49	63	0	37	0.01 c	14 a	2 ab
8 - 0.25[6]	39	77	0	23	0.02 a	18 a	2 b

[1] Treatment: length in weeks and 2,4-D concentration in mg.l^{-1}, for example: 1 - 4.0 = one week culture on medium containing 4.0 mg.l^{-1} 2,4-D

[2] Number of callus pieces obtained from 60 excised embryos

[3] I = Compact callus, II = Globular callus, III = Soft callus, IV= Gelatinous callus. Callus type III and IV pooled.

[4] Callus weight per excised embryo at the end of the callus maintenance period on callus growth medium. No. of shoots and shoot primordia per excised embryo after 9 weeks on regeneration medium (MS+ 1 mg.l^{-1} kinetin).

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

[6] Control: 8 weeks of culture on medium containing 0.25 mg.l^{-1} 2,4-D

The results (Table 4) revealed that a period of one week with either 4 or 8 $mg.l^{-1}$ 2,4-D resulted in more compact and globular callus than in the control. The amount of callus was not influenced by treatments with relatively high 2,4-D concentrations. These high 2,4-D concentrations resulted in increased numbers of shoots per callus, as compared to the control (not significant). However, 2 weeks of 8 $mg.l^{-1}$ 2,4-D, followed by 6 weeks of 0.25 $mg.l^{-1}$ 2,4-D resulted in significantly more regenerated shoots than in the control. The number of shoot primordia was not influenced by a temporary high 2,4-D concentration.

Morphological and histological observations.

Callus development started within three days after transfer of the isolated mature embryos to callus induction medium. At the place of the shoot apex and sometimes at the remaining cotyledon, a thickening could be observed within one week of culture (Fig.5). Histological observations revealed that the callus tissue was produced by the shoot meristem and the cotyledon part (Fig.6). Four types of callus with varying morphogenetic potential, as earlier described, were obtained.

Plant regeneration became visible one week after transfer of callus pieces to regeneration medium. Both lobate and globular structures produced chlorophyl. More globular structures were produced on regeneration medium. Development of new meristematic regions within lobate and globular structures occurred (Fig.7). These meristematic regions developed into root and shoot meristems. Anatomically, the somatic embryos (Fig.8) were bipolar, possessed root and shoot meristems and resembled the zygotic embryo. Simultaneous development of root and shoot was observed only infrequently. Somatic embryos were formed asynchronously and various stages of development were observed. Most of the embryos were formed in clusters with their cotyledons fused together. Four weeks after transfer to regeneration medium numerous green cotyledons could be observed and within eight weeks roots were formed at the bases of the cotyledons and primary leaves broke through a slit in the cotyledon (Fig.9). Adventitious shoots were formed directly from meristems which arose continuously on the surface of the callus. Newly formed plants could be easily separated and were transferred to soil with hardly any losses (Fig.10).

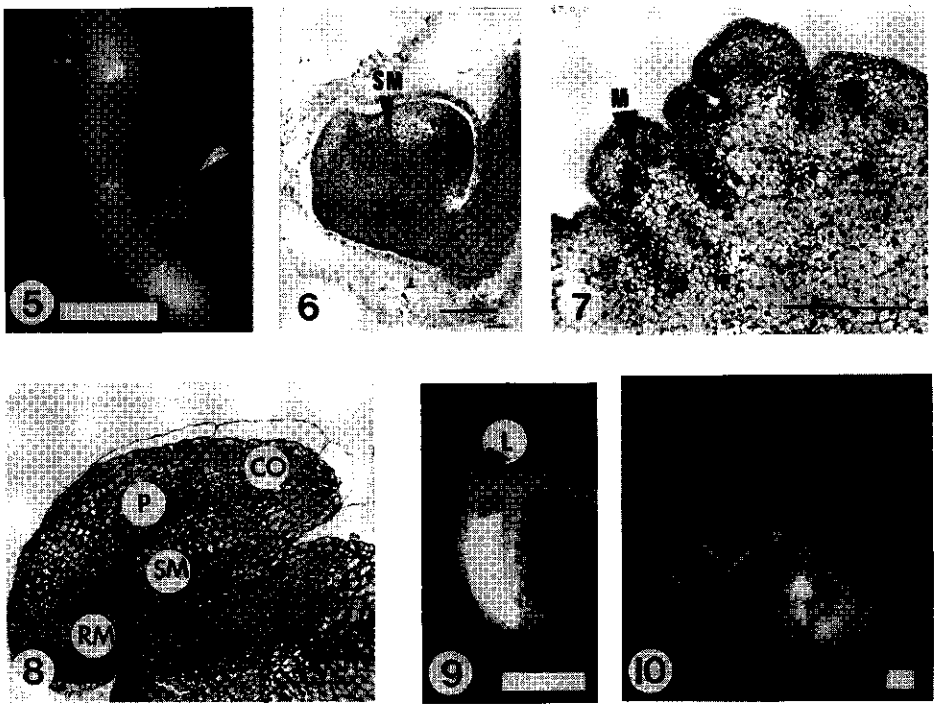


Fig. 5. Callus formation (arrow) at the place of the shoot base of leek (Bar = 2.5 mm). **Fig. 6.** Longitudinal section through a zygotic embryo after 2 weeks on callus induction medium with 1 $mg.l^{-1}$ 2,4-D (Bar = 1.0 mm). **Fig. 7.** Transverse section of leek with developing globular structures and meristematic regions on callus growth medium with 1 $mg.l^{-1}$ 2,4-D. **Fig. 8** Longitudinal section through a young somatic embryo of leek on regeneration medium with 1 $mg.l^{-1}$ kinetin (Bar = 1.0 mm). **Fig. 9.** Fused leek embryos with bent cotyledons, roots and developing primary leaves. **Fig. 10.** Formation of leek plantlets after 8 weeks on regeneration medium. CO = cotyledon, M = meristematic region, SM = shoot meristem, RM = root meristem, P = procambial strand, L = primary leaf.

Discussion

In leek, four different callus types have been morphologically distinguished. Compact and globular callus showed a good shoot regeneration, in contrast with soft and gelatinous callus types. Van der Valk et al. (1992) and Buiteveld et al. (1993) only distinguished between two callus types: a watery, friable and a dry, compact and lobate type and they also concluded that watery and friable types possessed a low regeneration ability.

Callus types were sometimes heterogeneous and consisted of a mixture

of different types. With increasing age the callus type could change. Globular structures developed on the surface of compact callus after prolonged culture. It is, therefore, assumed that this callus type originates from the compact callus type: the most common type in our study. According to Novák (1990), *Allium* species have a strong tendency to form compact callus. In cereals, Vasil (1985) distinguished between "embryogenic" (E) callus and "non-embryogenic" (NE) callus. The NE callus was yellow to translucent, wet, rough to crystalline in appearance and resembled our soft and gelatinous callus. E callus had a smooth, white knobby appearance and resembled our compact and globular callus. E callus frequently gave rise to plants by somatic embryogenesis; while NE callus occassionally gave rise to shoots and roots by organogenesis. Nabors et al. (1983) also showed that E callus produces NE callus and vice versa.

Our results show that a high shoot regeneration can be obtained in mature embryo derived callus cultures of leek. The average number of regenerated shoots per cultured embryo was strongly influenced by the genotype of the explant material used and by culture conditions and varied in our experiments from 1 to 9. Van der Valk et al. (1992) obtained an average of 5 to 9 and Buiteveld et al. (1993) of 0-7 shoots per callus per genotype. The genotypic factor was investigated more into detail by Schavemaker and Jacobsen (1995).

Shoot regeneration and primordia formation were influenced strongly by the callus type. This is in accordance with previous reports on *Allium* species (Dunstan & Short 1977; Van Doorne et al.1988).

Callus induction and growth in *Allium* species are strictly dependent on the presence of exogenous auxins (Novák 1990). Van Doorne et al. (1988) induced callus on germinating seeds of leek and obtained 10-30 shoots per genotype. They concluded that the number of regenerated shoots is not influenced by the callus weight and the composition of the media. In our research, we observed that higher 2,4-D concentrations decreased the percentage of regenerative callus types (compact and globular) as well as the number of regenerated shoots and shoot primordia of the specific callus types. Therefore, the decrease in shoot regeneration and primordia formation at high 2,4-D levels can be explained by a decrease in regenerative callus types and by a decrease in shoot and primordia production of these specific callus types.

Low 2,4-D levels (0.25 and 0.5 $mg.l^{-1}$) resulted in a better shoot regeneration for leek. The negative effect of 2,4-D on shoot regeneration was also observed for onions (Shahin & Kaneko 1986; Dunstan & Short 1978; Phillips

& Luteyn 1983).

We further showed that the length of the callus maintenance period had a negative effect on the regenerative ability of a callus culture. In general, the regenerative potential of callus can be lost after repeated subcultures (Pierik 1987). A callus growth period of 12 weeks should be considered as too long. This phenomenon was also observed for onion by Dunstan & Short (1978) and for leek by Van der Valk et al. (1992). The callus growth period for leek can be reduced to 6 weeks, for an optimal shoot regeneration. A short callus induction period with a relatively high 2,4-D concentration followed by a prolonged callus proliferation period at low 2,4-D concentrations (0.25 $mg.l^{-1}$) is advisable. An alternative is the development of a cyclic regeneration system as demonstrated by Schavemaker and Jacobsen (1995).

According to Novák (1990), several authors reported plant regeneration from callus cultures of *Allium* as "somatic embryogenesis", without giving clear morphological and histological evidence. There is some positive morphological evidence for this process in *A. carinatum* (Havel & Novak 1988), *A.cepa* and hybrids between *A. fistulosum* and *A.cepa* (Lu et al. 1989; Van der Valk et al. 1992), *A. rosenbachium* (Keller 1992) and *A.porrum* (Van der Valk et al. 1992; Buiteveld et al. 1993). Histological evidence for the occurence of somatic embryogenesis was not given by these authors. The most distinctive characteristic of an embryo is its anatomically discrete (closed) radicle end (Haccius 1978). Examinations of the radicle end of the plantlets induced in culture enables us to distinguish between somatic embryos and buds. A somatic embryo consists of a very young shoot meristem at one side of the embryo axis and a root meristem at the other side. Vascular cells connect the two meristems and no callus is present between them (Emons & De Does 1993). Somatic embryos with opposite shoot and root meristems have been found in our histological study. Apart from somatic embryos, bud-like structures were visible.

Many tissue culture systems are known where both embryogenic callus and shoot buds were produced from the same culture. Explants can be induced to follow different morphogenetic pathways. (Bhaskaran & Smith 1990) . Havel & Novák (1988) observed that callus derived from various plant parts was characterized by different regeneration patterns. It is obvious that plant regeneration in callus cultures derived from zygotic leek embryos also follows different pathways, indicating that both organogenesis and somatic embryogenesis occur.

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CHAPTER 7

Genetic variation and control of plant regeneration in leek (*Allium ampeloprasum* L.)

B. Silvertand, E. Jacobsen and A.M van Harten

The Graduate School of Experimental Plant Sciences, Department of Plant Breeding, Wageningen Agricultural University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands

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Summary. Zygotic embryos of leek (*Allium ampeloprasum* L.) were isolated from mature seeds of different cultivars, selfings and full-sib families. The embryos were cultured on callus induction and shoot regeneration medium and employed to study several parameters: percentage of embryos forming calluses, percentage of embryos forming compact calluses, callus weight, percentage of regenerating calluses, numbers of shoot primordia and numbers of regenerated shoots. Differences between cultivars and selfings were found for most parameters studied. For all cultivars all parameters, except callus weight, decreased after one generation of selfing. Compact callus types enhanced primordia formation and shoot regeneration. Genetic characteristics of callus development and plant regeneration were studied in a 4x4 diallel cross. Analysis of variance revealed significant differences between full-sib families. The diallel analysis showed that additive gene effects were significant for all parameters. The predominance of additive gene effects indicated high narrow-sense heritability. Breeding for an increased number of regenerated shoots was successful.

Introduction

Leek (*Allium ampeloprasum* L.) is an autotetraploid (2n=4*x*=32), mainly outbreeding and monocotyledonous crop, grown for its edible (false) stem. Every leek genotype is represented only by a single plant, and leek cultivars are, therefore, heterogeneous and unstable. Much effort is being made in the Netherlands to improve uniformity by breeding hybrid leek varieties.

Tissue culture technologies can be helpful tools in leek breeding programmes. *In vitro* multiplication techniques via organogenesis have been described by several authors and offer possibilities for clonal propagation of selected leek genotypes (reviews see Novák et al. 1986a; Novák et al. 1986b and Novák 1990). Callus culture and regeneration from callus by organogenesis and somatic embryogenesis have also been described for leek (Van der Valk et al. 1992; Buiteveld et al. 1993; Silvertand et al. 1995; Schavemaker and Jacobsen 1995). These offer possibilities for somatic hybridization, genetic transformation and mutant selection. In a previous paper the presence of significant variation among genotypes for callus formation and plant regeneration was reported (Silvertand et al. 1995), and it was suggested that callus development and plant regeneration are under genetic control. It is frequently observed that regeneration *in vitro* varies considerably among species or cultivars, and among individual genotypes within a species (Halperin 1986; Bhaskaran and Smith 1990).

Detailed information on the inheritance of competence for callus development and plant regeneration in leek has not been reported. The aim of this study was to evaluate the mode of the genetic control of callus growth and plant regeneration in leek and to establish genotypes with improved *in vitro* shoot regeneration.

Materials and Methods

Plant material. Seeds from seven commercial leek cultivars (Castelstar, Gavia and Snowstar from Enza Zaden, Enkhuizen; Porino and Farinto from Nunhems Zaden, Haelen and Dakota and Strata from Royal Sluis, Enkhuizen) were used. Selfings of different genotypes of these cultivars were performed. Four genotypes (Dak-1, Far-2, Cas-3, Cas-4) were selected with reference to the results of previous experiments, in which these genotypes showed differences in competence for plant regeneration. Every genotype was represented by four *in vitro* cloned plants, originating from one zygotic embryo. Dak-1 was derived from the cultivar Dakota, Far-2 from Farinto and Cas-3 and Cas-4 from Castelstar. A full set of diallel crosses was performed among the four genotypes.

Explants. Mature seeds from cultivars, selfings and full-sib families were surface sterilized for 30 sec in 70% ethanol and 20 min in 1% sodium hypochlorite (commercial bleach) with a drop of Tween 20 and subsequently rinsed four times (5, 10, 15 and 20 min) in sterile water. After sterilization the mature zygotic embryo was isolated from the seed.

Media and Culture Conditions. For callus induction, isolated embryos were placed on callus induction medium, consisting of MS salts and vitamins (Murashige and Skoog 1962), 30 $g.l^{-1}$ sucrose, 2 $mg.l^{-1}$ 2,4-D (2,4-dichlorophenoxy acetic acid) and 8 $g.l^{-1}$ agar (Daishin, Brunschwig). Media were adjusted to pH 5.8 prior to autoclaving for 20 min. Cultures in Petri dishes (4 cm diameter) were incubated in the dark at 21°C and calli were subcultured at three week intervals on the same medium. For regeneration, calli (9 weeks) were transferred to MS medium, supplemented with 30 $g.l^{-1}$ sucrose, 8 $g.l^{-1}$ agar and 1 $mg.l^{-1}$ kinetin and cultured in Petri dishes (4 cm diameter). Cultures were placed in the light, approx. 20 $W.m^{-2}$, (16/8 h photoperiod) at 21°C and subcultured at three week intervals on the same medium. After the first culture (3 weeks), these calli were transferred to glass jars.

Experiments and Statistical analysis. Different parameters for callus formation and regeneration were studied. The percentage of embryos that formed callus was determined after three weeks of culture. Following the reports of Van der Valk et al. (1992) and Buiteveld et al. (1993) a distinction was made in our experiments between the compact and the soft type of callus. The percentage of embryos forming compact calluses was recorded. Callus weight per excised embryo was determined at the end of the callus growth period (9 weeks). After a nine week regeneration period, green shoot primordia were observed and counted. Regenerated plantlets measuring more than 5 mm were counted and classified as shoots. The percentage of initial calluses that developed shoots was recorded as the percentage of regenerating calluses. No distinction was made between plantlets obtained via organogenesis or somatic embryogenesis. Calluses were not split during the callus growth and regeneration period and the data can be traced back to one excised embryo.

In a first experiment, mature zygotic embryos (36 per cultivar) derived from six different cultivars, and mature zygotic embryos derived from individual plants of I_1 progenies from the six cultivars, were compared for the above-mentioned six parameters. Group means were compared by calculating Student's *t* value ($P \leq 0.05$).

The diallel experiment was a randomized complete block design with six replications and six embryos per Petri dish as one experimental unit. The means of the 12 full-sib families were used in the diallel analysis according to method 3, model I of Griffing (1956).

Results

Analysis of cultivars and selfings

The six cultivars showed significant differences in callus weight, percentage of regenerating calluses and number of shoot primordia (Table 1). No significant differences were found between the six cultivars in the percentage of embryos that formed calluses, percentage of compact calluses and number of regenerated shoots. Porino showed the highest percentage of embryos that formed calluses (92%), percentage of compact calluses (84%) and number of shoot primordia (26), but exhibited the lowest percentage of regenerating calluses. Castelstar showed a low callus weight (31 mg) after nine weeks of callus growth. Gavia gave the highest number of regenerated shoots (10) and percentage of regenerating calluses (79%). Strata exhibited the highest callus

weight (65 mg) and Dakota gave the lowest number of regenerated shoots (6).

After one generation of selfing, significant differences between the six cultivars were found for all parameters (Table 1). Porino had the lowest percentage of embryos that formed calluses (33%) and percentage of compact calluses (25%). Castelstar again showed a low value for callus weight (40 mg) and Snowstar exhibited a low percentage of regenerating calluses (30%). Strata and Dakota demonstrated the highest percentage of embryos that formed calluses (83% and 82% resp.). Dakota had the highest percentage of compact calluses (65%), percentage of regenerating calluses (57%), number of shoot primordia (12) and number of regenerated shoots (8).

One generation of selfing resulted in a significant ($P \leq 0.01$) decrease in the percentage of embryos that formed calluses, percentage of compact calluses, percentage of regenerating calluses, number of shoot primordia and number of regenerated shoots for most of the tested cultivars. However, the callus weight was not reduced after selfing.

Differences in response to selfing between cultivars were observed. Porino showed a large decrease in the percentage of embryos that formed calluses, percentage of compact calluses, number of shoot primordia and number of regenerated shoots when compared to the other cultivars. The percentage of regenerating calluses and number of regenerated shoots in Gavia were reduced drastically after selfing. The other cultivars exhibited a less severe reduction for all parameters and Dakota showed hardly any decrease for all parameters after selfing (Table 1). The low number of shoot primordia and regenerated shoots for this cultivar did not further decrease after selfing. In general, cultivars with high values for a parameter showed a severe reduction of that value after selfing, while cultivars with an average or low value for a parameter showed a less reduced or stable value on selfing.

Correlation coefficients between all pairs of the parameters were calculated (Table 2). The percentage of embryos that formed calluses had no significant influence on any of the other parameters. Significant correlation coefficients were found between the following pairs of parameters: percentage of compact calluses and percentage of regenerating calluses; percentage of compact calluses and number of shoot primordia; and percentage of compact calluses and number of regenerated shoots. An increase in the percentage of compact calluses resulted in an increase in the percentage of regenerating calluses, number of shoot primordia and number of regenerated shoots. No significant correlation coefficients were found between the following pairs of parameters: callus weight and number of shoot

primordia; and callus weight and number of regenerated shoots. A high callus weight did not result in an increased percentage of regenerating calluses, number of shoot primordia and number of regenerated shoots. As expected, significant correlation coefficients were found between the percentage of regenerating calluses and number of shoot primordia and between the percentage of regenerating calluses and number of regenerated shoots. Finally, a high number of shoot primordia resulted in a high number of regenerated shoots. Additional analysis of the data revealed that the percentage of compact calluses had the largest positive influence on the percentage of regenerating calluses, number of shoot primordia and number of regenerated shoots. Thus, a high percentage of compact callus provided the best guarantee for a high shoot regeneration.

Table 1. Mean values for percentage of embryos that formed calluses (PECA), percentage of compact calluses (PCCA), callus weight (CAWE) in mg, percentage of regenerating calluses (PRCA), number of shoot primordia (NSHP) and number of regenerated shoots (NSHO) for six leek cultivars and for the same six leek cultivars after one generation of selfing.

Cultivar	(N)[1]	PECA	PCCA	CAWE	PRCA	NSHP	NSHO
Cultivars							
Porino	36	92 a[2]	84 a	41 ad	53 a	26 a	8 a
Castelstar	36	81 a	79 a	31 ac	55 ac	24 ac	8 a
Gavia	36	78 a	66 a	55 bd	79 bc	25 a	10 a
Snowstar	36	78 a	71 a	47 bd	57 ac	15 bc	8 a
Strata	36	86 a	72 a	65 b	68 ac	19 ac	9 a
Dakota	36	83 a	75 a	53 b	61 ac	13 bc	6 a
Selfings							
Porino	36	33 a[2]	25 a	62 ac	50 a	7 a	2 a
Castelstar	239	69 b	54 ac	40 a	45 a	7 a	5 a
Gavia	215	71 bd	56 ac	47 ac	39 b	7 a	3 a
Snowstar	147	61 b	49 bc	55 bc	30 b	6 a	2 a
Strata	110	83 c	51 ac	55 ac	45 a	6 a	2 a
Dakota	60	82 cd	65 bc	55 bc	57 a	12 b	8 b

[1] Number of embryos cultured.

[2] Within each column treatments with different letters are significantly different at the 5% level (t-test)

Table 2. Correlation coefficients for all pairs of parameters for percentage of embryos that formed calluses (PECA), percentage of compact calluses (PCCA), callus weight (CAWE), percentage of regenerating calluses (PRCA), number of shoot primordia (NSHP) and number of regenerated shoots (NSHO) for leek.

	Correlation Coefficients					
	PECA	PCCA	CAWE	PRCA	NSHP	NSHO
PECA		0.20	-0.09	-0.01	O.02	0.06
PCCA			0.01	0.36*[1]	0.42*	0.34*
CAWE				O.O1	0.05	0.01
PRCA					0.44*	0.58*
NSHP						0.52*

[1] *, Significant at the 1% level (t-test)

Analysis of the diallel cross

Four *in vitro* propagated genotypes (Dak-1,Far-2, Cas-3 and Cas-4), with varying *in vitro* shoot regeneration, were further analysed in a 4x4 diallel cross, resulting in 12 full-sib families and four selfings. The numbers of regenerated shoots per callus were the only available data from the parents. Genotype Dak-1, Far-2, Cas-3 and Cas-4 regenerated 21, 6, 15 and 9 shoots respectively.

Most hybrids showed midparent values or less for the number of regenerated shoots (Table 3). The cross between the two parents with the highest number of regenerated shoots (Dak-1 and Cas-3) exceeded the best parent and resulted in the highest number of regenerated shoots (23). However, the reciprocal of this cross only resulted in 14 shoots. On the contrary, the cross between the two worst parents (Far-2 and Cas-4) resulted in the lowest number of regenerated shoots (4). In general, differences between reciprocal crosses were observed for all parameters. The diagonal axis gives information about the selfings. The percentage of embryos that formed calluses of the selfings of Dak-1, Far-2 and Cas-3 was not lower than that of the corresponding full-sibs. In our previous experiments with different cultivars we found that the percentage of embryos that formed calluses decreased due to selfing. The values for the percentage of compact calluses, percentage of regenerating calluses, number of shoot primordia and number of regenerated shoots were lower for the four selfings than for the corresponding full-sibs and in agreement with the results from our previous experiment. The callus weight values of the selfings with Far-2 and Cas-4

were comparable with the average callus weight values of the full-sibs. However, Dak-1 showed a reduced callus weight, whereas Cas-3 exhibited an increased callus weight after selfing. The reduced callus weight for Dak-1, was rather unexpected. In our first experiment we noticed that genotypes derived from Dakota showed no reduced values for all parameters after selfing. Cas-3 was derived from Castelstar and in our cultivar experiment we also noticed that the callus weight of Castelstar increased after selfing.

Table 3. Mean values per parent for number of regenerated shoots (NSHO; f) and mean values per full-sib family for percentage of embryos that formed calluses (PECA; a), percentage of compact calluses (PCCA; b), callus weight (CAWE; c) in mg, percentage of regenerating calluses (PRCA; d), number of shoot primordia (NSHP; e) and number of regenerated shoots (NSHO; f) for a 4x4 diallel set of crosses in leek.

	Female			
Male	Dak-1 f 21	Far-2 f 6	Cas-3 f 15	Cas-4 f 9
Dak-1 f 21	a 83 d 60 b 72 e 8 c 36 f 12	a 71 d 47 b 59 e 10 c 96 f 7	a 92 d 85 b 94 e 16 c 71 f 14	a100 d 75 b 72 e 16 c 79 f 20
Far-2 f 6	a 75 d 67 b 62 e 13 c 33 f 13	a 86 d 81 b 68 e 16 c 47 f 6	a 75 d 52 b 59 e 11 c 56 f 12	a100 d 61 b 72 e 13 c 25 f 4
Cas-3 f 15	a 61 d 82 b 82 e 39 c 51 f 23	a 97 d 54 b 75 e 12 c 31 f 4	a 97 d 54 b 62 e 12 c 61 f 5	a 97 d 66 b 85 e 15 c 24 f 4
Cas-4 f 9	a 72 d 85 b 80 e 12 c 39 f 20	a 86 d 55 b 71 e 8 c 44 f 4	a 97 d 72 b 88 e 16 c 55 f 10	a 81 d 52 b 61 e 10 c 45 f 5

The first step in the diallel analysis was to test for differences among the 12 full-sib families. Highly significant F ratios were obtained for all parameters, indicating that family differences existed (Table 4). A significant block effect was found for percentage of compact calluses and significant family x block interactions were found for percentage of regenerating calluses.

Family effects were further partionated into general combining ability (GCA), specific combining ability (SCA) and reciprocal effects (REC) (Table

4). The diallel analyses showed that mean squares for general combining ability effects were significant ($P \leq 0.01$) for all parameters studied. Specific combining ability effects and reciprocal effects were significant ($P \leq 0.01$) for number of shoot primordia and number of regenerated shoots. Reciprocal effects were also significant ($P \leq 0.01$) for percentage of embryos that formed calluses and callus weight. Furthermore high GCA:SCA ratios were found for all parameters except for number of shoot primordia.

General combining ability, specific combining ability and reciprocal effects were calculated for the four genotypes (data not shown). A high GCA value for percentage of embryos that formed calluses and percentage of compact calluses was found for Cas-4. Dak-1 showed a high GCA value for callus weight, percentage of regenerating calluses, number of shoot primordia and number of regenerated shoots. High SCA values for number of shoot primordia were found for crosses between Dak-1 and Cas-3 and Far-2 and Cas-4, while high SCA values for number of regenerated shoots were found for crosses between Dak-1 and Cas-4 and Far-2 and Cas-3. The callus weight was strongly influenced by REC effects. A cross between Dak-1 as female parent and Cas-3 as male parent resulted in the highest number of shoot primordia and number of regenerated shoots, because of high GCA effects for Dak-1 and strong REC effects between both genotypes.

Table 4. Analysis of variance involving 12 full-sib families of leek from a 4x4 diallel cross. Mean squares for percentage of embryos that formed calluses (PECA), percentage of compact calluses (PCCA), callus weight (CAWE) in mg, percentage of regenerating calluses (PRCA), number of shoot primordia (NSHP) and number of regenerated shoots (NSHO).

Source	df	Mean squares					
		PECA	PCCA	CAWE	PRCA	NSHP	NSHO[1]
Families	11	6793*[1]	4631*	18317*	6250*	2243*	1759*
GCA	3	10179*	9096*	18810*	18279*	3735*	4359*
SCA	2	63	252	741	2169	1677*	1088*
REC	6	7323*	2604	23748*	1755	1743*	651*
Blocks	5	713	9671*	789	2619	468	223
FamxBlock	55	1293	2193	1639	3399*	420	161
Error (σ^2)	360	1057	1569	1341	1905	335	151
SCA:GCA		1:162	1:36	1:25	1:8	1:2	1:4

[1] *, Significant at the 1% level (t-test).

Discussion

No significant differences between the six cultivars were found for the percentage of embryos that formed calluses, percentage of compact calluses and number of regenerated shoots. Callus weight, percentage of regenerating calluses and number of shoot primordia differed among the cultivars. Differences between cultivars in these parameters became more pronounced after one generation of selfing. Inbreeding depression resulted in a decreased number of shoot primordia and number of regenerated shoots. Cultivar differences in shoot production were also examined by Baumunk-Wende (1989). Interactions with respect to *in vitro* propagation were reported between cultivars, between cultivars and their inbred lines, and between inbred lines of various generations. Inbreeding resulted in reduced callus weight and shoot production. Callus weight was not reduced in this study, however selfed progenies showed slightly higher callus weight than the original cultivars. Van der Valk et al. (1992), Buiteveld et al. (1993) and Silvertand et al. (1995) reported that compact calluses lead to a higher shoot regeneration than soft calluses. This positive correlation between the percentage of compact calluses and subsequent primordia and shoot production was confirmed by this study. A high callus weight, on the other hand, turned out to be no guarantee for high primordia and shoot production. Genotypic differences in formation of compact calluses, callus weight and shoot production were also observed by the same authors. A large genotypic difference within cultivars was observed in this study for all parameters studied.

Our analysis of variance revealed significant differences between full-sib families for all parameters. A correlation between genotype and *in vitro* response, particularly regeneration, has been reported in several species (Vasil 1986). Schavemaker and Jacobsen (1995) were able to select full-sib families of leek with improved *in vitro* somatic embryo production. They showed that selection of the best somatic embryo producing genotype had a positive effect on callus formation of these somatic embryos. It remains questionable, whether the influence of the genotype on plant regeneration is not overestimated and only related to endogenous hormone levels. According to Vasil (1986) and Bhaskaran and Smith (1990), the physiological and developmental states of the explants exercise more control on its morphogenetic competence than its genotype. It seems, however, that the total morphogenetic competence is influenced by a combination of genetic and environmental factors. Improvement of the genetic basis for morphogenetic competence is an impor-

tant factor and its role should not be underestimated.

The genetics of plant regeneration *in vitro* is complex (Carman 1990). Results from several species indicated that plant regeneration was genetically controlled by either nuclear or cytoplasmic genes or both (Bhaskaran and Smith 1990).

Combining ability analysis demonstrated the predominance of general combining ability effects for all parameters in our study. The GCA mean squares were several times larger than the SCA mean squares, indicating a considerable magnitude of additive gene effects. Tomes and Smith (1985), Ou et al.(1989) and Quimio and Zapata (1990) reported, for several other species, control of callus formation and plant regeneration by additive gene effects. Callus weight in this experiment was affected strongly by reciprocal effects. A further study revealed that callus weight was mainly controlled by maternal effects. The genetic analysis showed a strong negative maternal effect for Dak-1 on callus weight. This maternal effect was also observed in maize by Tomes and Smith (1985). The origin of this effect remains unknown, but could be influenced by cytoplasmic factors of the parent (e.g. mtDNA), the physiological characteristics of maternal plants or specific interactions between cytoplasmic and nuclear genetic factors (Tomes and Smith 1985; Narasimhulu et al. 1989; Sági and Barnabás 1989).

Shoot primordia formation and shoot regeneration were also controlled by SCA effects, indicating that dominant gene effects were also involved in the inheritance of these parameters. Abe and Futsuhara (1991) studied plant regeneration in rice and found that both dominance and additive effects were important for plant regeneration. The significance of reciprocal effects for number of shoot primordia and number of regenerated shoots suggested that not only nuclear genetic factors but also cytoplasmic factors or other maternal factors were involved.

The significant block effect for percentage of compact calluses can be explained by supposing an interaction between callus pieces within particular Petri dishes, resulting in an accumulation of compact callus pieces within these Petri dishes. A predominance of additive gene effects indicates high narrow-sense heritability. Consequently, effective selection should be possible within the F_1 and subsequent generations for all parameters. Thus, selection of parents on the basis of GCA effects seems advisable for improving *in vitro* callus development and plant regeneration of leek. Selection and breeding for a high shoot regeneration turned out to be successful in our study. Parents with a high shoot regeneration gave progenies with a high shoot regeneration. Furthermore, the significant SCA effects for number of shoot primordia and

number of regenerated shoots indicate that inbreeding depression as well as heterosis can be expected.

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CHAPTER 8

General discussion

Increasing interest in hybrid leek breeding has made pollination control via male sterility an important objective. The utilization of male sterility in the breeding of leek is still being hampered by the absence of a suitable genetic system for the maintenance of the male sterility trait. Leek breeders and researchers, in general, share the opinion that a cytoplasmic male sterility (CMS) system would be the best economically suitable and acceptable option for the production of hybrid leek. The race to introduce a CMS trait in leek has started only recently.

Mutation breeding technology, despite considerable efforts, sofar turned out to be unsuccessful. The question can be raised whether mutation breeding is the first and best approach to be followed under the present circumstances, in order to obtain the desired trait. To my opinion it is a good alternative, but not the first one to be used. Sofar, the most successful CMS systems have been found in the wild or in crosses within a group of cultivars. According to Pearson (1981) CMS would probably be found, even though rarely, in all crops if they were searched intensively. A first and essential step in the establishment of CMS in leek, therefore, should be the search for CMS within existing leek cultivars or within its relatives. However, the idea of screening leek populations for male sterility and subsequently performing testcrosses, apparently, does not fit in the modern line of thought about plant breeding research. But why artificially creating CMS in a lab, while nature already supplied us with this trait or with the basic material to create this trait? The rule to solve a problem should be 'simplicity'.

An extensive screening of leek populations and its relatives within the *Allium ampeloprasum* L. complex for male sterility combined with a search for differences in the mtDNA should be the primary step. Male sterile plants spontaneously arise in leek cultivars and it seems worthwhile to examine leek cultivars and "wild" populations within the *Allium ampeloprasum* complex for male sterile plants and to find a genetic explanation for this male sterility trait. It must be determined whether the male sterility is caused simply by segregating nuclear male sterility genes (NMS) or by segregating nuclear restorer genes in a sterilizing cytoplasmic background. Couvet et al. (1990) proposed that male sterility was caused by changes at the level of cytoplasmic genomes, coupled with the dynamics of nuclear and cytoplasmic gene pools in gynodioecious species.

Leek can probably be regarded as a gynodioecious species, because of the maintenance of male sterile plants in the population. In nature, the existence of two sex phenotypes, hermaphrodites (i.e. flowers with both stamens and pistels) and male steriles, is a constant feature of gynodioecious

plants. This sex dimorphism is genetically conditioned and is stable in many species. Two to fifty percent of male sterile plants are found in stable gynodioecious populations of various species. For this a continuous and rigorous selection of male steriles is needed in these populations. The mode of inheritance is critically important because the conditions for maintenance of male sterility in natural populations are very different depending on whether nuclear or cytoplasmic genes are involved or both (Lewis 1941; Cosmides and Tooby 1981; Charlesworth 1981; Gouyon and Couvet 1985; Van Damme and Van Damme 1986; Stevens and Van Damme 1988; Frank 1989; Couvet et al. 1990; Gouyon et al. 1991; Saumitou-Laprade et al. 1994). In the case of nuclear inheritance, male sterile plants must be more than twice as fecund as hermaphrodites (Lewis 1941). If both nuclear and cytoplasmic genes are responsible for male sterility, i.e. if the inheritance is nucleo-cytoplasmic, the relationship between frequency of females and their relative fecundity is the same as in the case of nuclear inheritance (Couvet et al. 1990). The inheritance is purely cytoplasmatic in case of absence of the restorer genes.

Following the theory of Gouyon et al. (1991) that stable maintenance of male sterility in populations of a gynodioecious species is based on an interaction between nucleus and cytoplasm, it can be proposed that the NMS system in leek is in fact a CMS system, and that the male sterility is caused by segregating nuclear restorer genes in a sterilizing cytoplasmic background. The nuclear restorer genes lead to identical segregation ratios for male sterility in leek progenies as the nuclear male sterility gene. Hermsen (1965) argued that, further elaborating out this theory, consistently one reaches the conclusion that cytoplasms which can restore fertility in male sterile lines may be expected to occur in nature. These "fertilizing" (N-) cytoplasms might be found either in normal varieties or in related species or genera. The maintenance of a male sterile genotype would be a simple procedure if such an N-cytoplasm could be discovered. Identification can be performed via a screening of the mitochondrial DNA. The presence of a relatively high percentage of male sterile plants in a leek population or related relatives can be a first indication that the male sterility trait is maintained by a nucleo-cytoplasmic inheritance and that, subsequently, a male sterility maintaining N-cytoplasm may be involved. This theory remains speculative for leek, until a sterility maintaining (N) cytoplasm is identified. The screening of the mtDNA of leek and its relatives has only recently been started. Two distinct cytoplasm types were identified within leek cultivars and more deviating types within relatives of leek (Silvertand et al. not published; Kik pers. comm.). The relevance of these different mtDNA types on behave of nucleo-

cytoplasmic male sterility in leek has to be investigated by intra- and interspecific crosses.

An alternative and very interesting approach of creating CMS in this respect is the selection of mitochondrial genome mutations (i.e. maintainer genotypes) in tissue culture systems, described by Hanson (1984), Earle et al. (1987), Rottmann et al. (1987), Bonhomme et al. (1991), Morère-Le Paven et al. (1992). Male sterile leek plants can be transferred to a tissue culture system and the regenerated plants examined for male fertility. The mtDNA of male fertile plants should then be compared with the mtDNA of the original male sterile plants. The male fertile plants subsequently can be used as pollinators for their male sterile counterparts. Maintainer genotypes can be identified by the segregation ratios in the progenies of the male sterile plants.

The tissue culture approach offers some considerable advantages as compared to the convential mutation breeding techniques. According to Karp and Bright (1985) and Dikalova et al. (1993) the environmental stress may act as a 'genomic shock' and an inducer of rearrangements in the mitochondrial genome during in vitro cell culturing. The tissue culture environment may turn out to be a better inducer of mutations in the mtDNA than chemical or physical mutagens. Palmer and Herbon (1988) predict that plant mitochondria are likely to contain highly efficient systems for repairing damage to their DNA because they show low rates of point mutations. These systems may restore the effects of chemical and physical mutagens. Another advantage of a tissue culture system is the high number of individuals simultaneously put under selection; especially if single cell units can be used. The regeneration pathway via single cells leads to an increased chance for identification of the mutation in the first generation after tissue culture. In the conventional mutation breeding approach the mutation also occurs in single cells but in multi-cellular organisms, and the mutated area is not likely to occur in the germ line. Furthermore, the cytoplasmic type is manifested only in the M_2, M_3 or later generations. Another advantage is the easier identification of a male fertile plant among male steriles than vica versa. In conclusion it can be argued that the tissue culture approach increases the specificity and efficiency of the "mutation" and selection process as compared to the conventional mutation breeding technology. In vitro culture in combination with mutagenesis could, probably, be an interesting way to create and exploit somaclonal variation for use in plant breeding.

Male sterile leek plants were obtained among male fertile plants of the same clone, after shoot regeneration in callus cultures, in our experiments.

The eventual utilization of this male sterility requires that it can be transmissible in sexual crosses. The progenies of these male sterile plants were analysed and it was concluded that the male sterility trait was determined by a nuclear monogenic recessive inheritance. These preliminary results indicate that plant regeneration via somatic embryogenesis or organogenesis in leek, after long-term callus culture, may offer opportunities for the exploitation of somaclonal variation, e.g. mtDNA variation in future leek breeding research. Induction of male sterility mutations through tissue culture recently has been reported in sorghum (Elkonin et al. 1994). Test-crosses with different genotypes revealed that this inheritance was either nuclear recessive or cytoplamic, depending on the genotype of the parent.

Recent advances show that NMS can be artificially induced by transfomation with an engineered chimeric gene consisting of an anther-specific promoter associated to a MS "deleterious" gene (Mariani et al. 1990). According to Lasa and Bosemark (1993) the engineered NMS system is to be preferred above a CMS sytem, because of the higher risks and costs of the CMS system. The introduction of this NMS system in leek, however, will run into some difficulties related to the transformation of this monocot. The particle bombardment method can be applied to circumvent these difficulties. The expression and stability of the engineered male sterility trait within this species, the necessity to use large amounts of selective herbicides in the field, as well as the uncertainty about the acceptance by the consumers of the transformed leek plant remain uncertain aspects. A conventional CMS system for leek therefore has to be preferred to my opinion.

Direct transformation of plant mitochondria has not been reported sofar. In the immediate future, transformation of higher plant mitochondria can be envisaged using the particle bombardment method (Medgyesy 1990; Vedel et al. 1994) in order to introduce new CMS genes into species without CMS. The only method successfully used to induce changes in the mt gene expression in higher plants is protoplast culture and, more generally, protoplast fusion (Vedel et al. 1994).

Somatic hybridization by protoplast fusion is expected to increase genetic variability by recombination of nuclear and cytoplasmic genomes, and to allow the transfer of desirable traits from one species to another (Vedel et al. 1994). CMS could be transferred from one parental species to the other within the genera *Nicotiana*, *Petunia*, *Brassica* and *Oryza*. Transfer of CMS through protoplast fusion is expeditious and requires only 6 to 9 months as against 3 to 6 years through backcrossing. Attempts to transfer CMS from onion (*Allium cepa*) to leek seem to be worthwhile. Regeneration and fusion

techniques for leek protoplast systems have to be developed or optimized in order to create somatic hybrids between onion and leek. Whether it is possible for the onion mitochondrial genome to coexist in the same cell with the nuclear genome of the distantly related leek species is still an open question. One can only speculate about the expected problems of poor cooperation between the nucleus and cytoplasm. This so-called nucleo-cytoplasmic incongruity is stronger when the fusion parents are less related and may result in a hybrid that shows poor growth and female sterility or that may not even survive (Wolters 1994). This incongruity may become a major obstacle in fusion experiments between leek and onion.

The only remaining alternative for hybrid leek production for the very near future seems to be the vegetative propagation of nuclear male sterile plants by *in vitro* micropropagation or by *in vivo* bulbil formation. Micropropagation has been used to only a very limited extent for agronomic crops, but quite extensively for horticultural and ornamental species, which are normally propagated vegetatively. The higher costs for the *in vitro* or *in vivo* propagation must be compensated by an increased hybrid seed price. An important prerequisite in this respect is the establishment of a tissue culture system that offers a high multiplication factor, or specific genotypes that show a high rate of bulbil formation, in order to multiply the male sterile female line. The described flower stalk method may be a useful method for large scale *in vitro* multiplication of male sterile plants on behave of hybrid seed production in leek. The successful introduction of the first leek hybrids in 1995 by Nunhems Zaden demonstrates that it is technically possible and economically feasible to produce a leek hybrid that is based on a vegetative multiplication step.

The high rate of inbreeding depression and the tetraploid nature will be an additional obstacle in the further hybridisation of leek. It will be difficult to breed homozygous parent lines. For practical breeding, inbreeding should not go beyond the I_1 level, because of the severe loss in seed set (Schweisguth 1970, Smith and Crowther pers comm). The availability of diploids could increase the number of strategic options open to leek breeders. Smith et al. (1991) first reported the production of diploids in leek by ovule culture. However, this success was not repeated in additional work.

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Summary

In this thesis the results are presented of a five year study aiming at the genetic change of the recalcitrant leek (*Allium ampeloprasum* L.).

Leek is a mainly outbreeding autotetraploid ($2n=4x=32$) species, grown for its edible false pseudostem formed by the leaf sheaths. The crop shows some major imperfections, e.g. lack in uniformity and severe disease problems. The presently applied breeding systems (mass and family selection) are unsuitable to solve these problems. The importance of leek is increasing annually and modern cultural methods demand an uniform crop with a high level of disease resistance. The system that can bring leek to higher agronomical standards seems to be hybrid leek breeding. Hybrid leek breeding is hampered, however, by the lack of an economically suitable system of producing male sterile lines for the hybrid seed production. The availability of a male sterility trait is the key to hybrid leek breeding and the establishment, maintenance and application of this trait are the main objectives of the present work.

A monogenic inheritance can be accepted as explanation for the genetic control of the male sterility trait selected in our experiments. The acceptance of this nuclear inheritance does not automatically rule out the possibility of a nucleo-cytoplasmic interaction. A nucleo-cytoplasmic inheritance may well serve to explain the high percentages of male sterile plants in some progenies of male sterile plants, but this assumption can not be proven unless a maintainer {(N)rfrfrfrf} genotype is identified.

Intra- and interspecific crosses, sofar, did not result in the establishment of new male sterile leek genotypes. In the future, more attention should be paid to this, potentially, important source of cytoplasmic male sterility.

Attempts have been made to induce cytoplasmic male sterility in leek using N-nitroso-N-methyl urea. Application of N-nitroso-N-methyl urea results in poor seedling emergence, a high rate of chlorophyl deficient seedlings and a high percentage of male sterile plants in the M_1 generation. The percentage of male sterile plants in the M_2 generation, however, did not differ from the control and no additional mitochondrial DNA variation was found in these male sterile plants.

A new, simple, efficient and rapid *in vitro* method for mass clonal propagation of leek plants, using small flower stalk 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 high multiplication factor makes this method suitable for the vegetative propagation of male sterile genotypes on behave of hybrid seed production in leek.

The lack of an efficient regeneration system for leek has slowed down the progress of its genetic modification. For application of somatic hybridization and genetic transformation techniques, callus regeneration techniques are required. A high frequency plant regeneration system via organogenesis and somatic embryogenesis was established with callus cultures derived from mature zygotic embryos of different leek genotypes. Male sterile leek regenerants appeared among male fertile regenerants of the same clone after callus culture of leek. The induction of mtDNA variation via long-term callus culture of leek might be an attractive alternative for the induction of CMS. Differences between leek genotypes in callus type, callus weight, primordia formation and shoot regeneration were observed. These differences became more pronounced after inbreeding. A predominance of additive gene effects for callus weight, primordia formation and shoot regeneration was found after a diallel analysis.

Hybridisation in leek on the basis of vegetative multiplication of nuclear male sterile lines seems at this moment the only practically applicable option. However, the search for a system based on cytoplasmic male sterility must and will be continued.

Samenvatting

De veredeling van prei, dat een tetraploid en voornamelijk kruisbevruchtend gewas is, staat aan de vooravond van grote veranderingen. Binnen afzienbare tijd kunnen de huidige rassen vervangen worden door hybride rassen. De problemen binnen de bestaande preirassen, m.b.t. geringe uniformiteit en toegenomen ziektedruk vragen om een drastische aanpak. Hybride rassen kunnen een oplossing bieden voor deze problemen. Met hybriden is het mogelijk om meer uniform plantmateriaal te leveren dat geschikt is voor moderne oogst en verwerking. Bovendien is het gemakkelijker om ziekteresistenties in te bouwen.

De veredeling van hybriden wordt echter bemoeilijkt door het feit dat er op dit moment nog geen economisch verantwoord genetisch systeem voorhanden is, waarmee volledig mannelijk steriele moederlijnen geproduceerd kunnen worden. Deze steriele lijnen zijn noodzakelijk als moederlijn voor de oogst van het hybride zaad. In het kader van dit onderzoek werd er daarom naar oplossingen gezocht om zo'n systeem voor prei te verwezenlijken. In eerste instantie werd er getracht om een systeem op basis van cytoplasmatische mannelijke steriliteit te induceren via mutatieveredeling. Preizaden werden behandeld met mutagentia en in de eerste generatie (M_1) en tweede generatie (M_2) na behandeling werd er naar mannelijk steriele planten gezocht. Terugkruisingen en onderzoek van het mitochondriale DNA wezen echter uit dat er geen gronden waren om aan te nemen dat er cytoplasmatische mannelijke steriliteit werd geïnduceerd.

Onderzoek aan splitsende nakomelingschappen van prei wees uit dat de gevonden mannelijke steriliteit in prei een monogeen recessieve eigenschap is. Het kan echter niet worden uitgesloten dat er binnen prei een vorm van cytoplasmatische mannelijke steriliteit aanwezig is. Het probleem ligt bij de identificatie van het instandhouder genotype.

Een methode die zeer geschikt is voor de vermeerdering van genetisch mannelijk steriele planten is de *in vitro* bloemsteelmethode. Met deze methode kan een relatief hoge vermeerderingsfactor worden verkregen, waardoor het mogelijk wordt om de moederlijn vegetatief te vermeerderen.

Gedurende dit onderzoek werd er een callus regeneratiemethode ontwikkeld die de basis legt voor genetische transformatie en somatische hybridisatie technieken, uiteindelijk gericht op het verkrijgen van een genetisch bruikbaar systeem voor de produktie van mannelijk steriele lijnen. Deze laatste methode biedt eveneens mogelijkheden voor het induceren van somaklonale variatie en in het bijzonder CMS in prei.

Nawoord

Het proefschrift is nu eindelijk af. Gedurende het afgelopen jaar heb ik mij vaak moeten verantwoorden voor de voortgang bij het schrijven van dit proefschrift. Het schrijven zelf was voor mij echter nooit een probleem en ik produceerde met behulp van mijn pc veel tekst. Ik ondervond echter de nodige en natuurlijk uitermate nuttige tegenstand van de potloden en pennen van mijn begeleiders, die al strepende mijn teksten keer op keer reduceerden en bekritiseerden.

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Curriculum vitae

Bernard Catharina Hubert Johanna Silvertand werd geboren op 9 april 1966 te Nieuwenhagen. Van 1978 tot 1984 volgde hij de Atheneum opleiding op het Eijkhagencollege te Schaesberg. In 1984 begon hij met zijn studie aan de Landbouwuniversiteit te Wageningen. Vanaf 1985 tot 1989 volgde hij de doctoraalopleiding plantenveredeling, met als hoofdvakken plantenveredeling en tuinbouw. Nog voor de afronding van zijn studie plantenveredeling werd hij door dr. ir. Ton van Harten gevraagd om, in het kader van een promotie onderzoek aan mannelijke steriliteit bij prei te gaan werken. Het onderzoek werd mogelijk gemaakt met de hulp van enkele Nederlandse zaadbedrijven. De belangrijkste resultaten van dit onderzoek worden beschreven in dit proefschrift. Vanaf 1 maart 1995 is hij werkzaam bij S&G Seeds in Enkhuizen als veredelaar en onderzoeker van .....prei.