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Callus induction and plant regeneration from explants of commercial cultivars of leek (*Allium ampeloprasum* var. *porrum* L.)

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Summary. Plant regeneration capacity was studied for 8 cultivars and 4 accessions of leek (*A. ampeloprasum* var. *porrum* L.). Compact callus was induced on embryo and leaf explants on three different media. The highest frequency of compact callus formation (up to 90%) was obtained when mature, zygotic embryos were cultured on MS medium, containing 30 g/l sucrose and 1 mg/l 2,4-D. Regeneration occurred through somatic embryogenesis on MS medium, supplemented with 1 mg/l kinetin. Plants could be regenerated from all cultivars and accessions tested. These cultivars and accessions could be classified into three groups with respect to shoot formation frequency. The results suggest a distinct influence of the genotype on the morphogenic response of leek embryo explants *in vitro*.

Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; MS - Murashige and Skoog (1962) medium; N_6 - medium from Chu *et al.* (1975); B_5 - medium from Gamborg *et al.* (1968); BDS - Dunstan and Short medium (1977).

Introduction

There is considerable interest in plant tissue culture techniques, such as somatic hybridization, genetic transformation and *in vitro* selection for mutants as means to complement the leek breeding program. For the successful application of most of these techniques the availability of an efficient procedure for plant regeneration from protoplasts or suspension cultures is a prerequisite.

In monocots, including *Allium*, it has proven difficult to culture and regenerate protoplasts that have been directly isolated from the plant (Novák 1990; Vasil 1983). Indeed, plant regeneration has only been successful when morphogenic suspension cultures were used as the starting material for isolation of protoplasts (Vasil 1988). Therefore, in our research, emphasis has been placed firstly on the initiation of callus cultures that are suitable for the establishment of morphogenic suspension cultures. Thereafter, a reliable system for protoplast regeneration can be established and subsequently a method for somatic hybridization or cybridization.

Plant regeneration from callus cultures derived from different explants has been studied using several *Allium* species (Fridborg 1971; Dunstan and Short 1977; 1978; 1979b; Phillips and Hubstenberger 1987; Seo and Kim 1988; Van der Valk *et al.* 1992). In leek, callus cultures have been successfully established from different explants, i.e., basal plates (Debergh and Standaert-De Metsenaere 1976; Dunstan and Short 1979a), floral heads (Novák and Havel 1981) and zygotic embryos (Van der Valk *et al.* 1992). Shoots have also been regenerated from such callus cultures. However, whether regeneration occurs through organogenesis or somatic embryogenesis has not been investigated in detail for leek.

To our knowledge only few data have been reported on the regeneration frequency from callus cultures of leek (Van der Valk *et al.* 1992). In this report we describe callus induction and plant regeneration from callus cultures of different cultivars and accessions of *A. ampeloprasum* var. *porrum*. The regeneration frequency, as well as the mode of regeneration, were determined. Different callus induction media were used and embryo and leaf explants from different cultivars were tested for their amenability to form morphogenic callus, in order to define the optimal conditions for callus induction and plant regeneration.

Materials and methods

Plant material. Seeds of 8 *Allium ampeloprasum* var. *porrum* L. cultivars and 4 accessions of *Allium ampeloprasum* from Israel, as listed in Table 3, were supplied by the Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Wageningen, The Netherlands.

Explants. Mature seeds were sterilized according to the method of Van der Valk *et al* (1992). The embryos were aseptically excised using a stereo microscope. That part of the embryo containing the radicle, part of the cotyledon, and including the shoot apex was cut off and used for culture. For callus induction from mature embryos, 8 cultivars and 4 accessions were used. Leaf explants were obtained from 20 to 30 mm plantlets that had previously been regenerated from mature embryo-derived callus cultures. Shoots were sliced into approximately 2 mm long

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sections, starting at the base of the leaf, and were referred to as explant number 1, 2 and 3, respectively. Explant 1 contained meristematic tissue. Ten genotypes of both 'Porino' and 'Tilina' were used for callus induction on leaf explants and, on average, 6 explants per genotype were cultured.

Media and culture conditions. For callus induction, mature embryos were cultured on MS medium (Murashige and Skoog 1962), supplemented with 1 mg/l 2,4-D, 30 g/l sucrose and 0.3% (w/v) phytagel (Sigma). The medium was autoclaved and the pH was adjusted to 5.8. Per cultivar, 225 embryos were cultured. Leek cultivars are open-pollinated populations, thus each embryo represents an individual genotype. The cultures were incubated in the dark at 25°C for 6 weeks, after which the formation of compact callus was assessed. Compact calli were then transferred to fresh MS medium and subcultured at 3-weekly intervals. To compare the tissue culture response of the embryo and leaf explants, the explants and calli were cultured under the same conditions.

For regeneration, 3 month old compact calli (5 x 5 mm) derived from 20 embryos of each cultivar were transferred to MS medium, supplemented with 1 mg/l kinetin (MSK) and cultured at 25 °C with a 16h photoperiod (ca. 3000 lux white fluorescent light). Generally, 6 calli were cultured per embryo, but when an embryo did not produce enough callus, fewer were cultured. The morphogenic response was determined on the basis of the formation of somatic embryos, which was assessed after 3 weeks of culture and on the basis of the regeneration frequency (the mean number of plantlets produced per callus), which was assessed after 8 weeks. Regenerated plantlets were transferred to glass culture jars with half strength MS, 20 g/l sucrose and without hormones. The plants were transferred to the greenhouse after 4 weeks.

In one experiment, embryos of the cultivar 'Tilina' were compared for callus induction on three basal media: MS, Gamborg's B_5 medium (Gamborg et al., 1968), modified by Dunstan and Short (BDS) (1977) and N_6 medium (Chu et al, 1975). As indicated, the media were supplemented with 2 or 3% sucrose. In this experiment 1 mg/l 2,4-D, 200 mg/l casein hydrolysate and 2.3 g/l L-proline were added to all media. Per medium, 20 explants were cultured.

Data analysis. Counts of embryos and shoots were analyzed according to a generalized linear model for count data (McCullagh and Nelder, 1989). Calculations were carried out by means of the Genstat computer program. To test the equality of the frequency of compact callus formation, Pearson's X^2 test was used.

Measurement of ploidy level. The ploidy levels of 30 shoots of 7 cultivars regenerated from both 3 and 10 month old calli were measured by flow cytometry. Nuclear suspensions were prepared by chopping leaf material in a nuclear isolation/staining buffer containing DAPI, as described by Verhoeven (1990). The mixture was then filtered through a 55 μm filter to remove debris. The nuclei were measured for DNA content with a Partec PAS-II flow cytometer (Verhoeven, 1990).

Results

Effect of explant type on compact callus formation

Independent of the explant type cultured, two morphologically different callus types could be distinguished: (1) a compact, white and nodular type with proembryogenic structures (Fig. 1A); and (2) a friable, watery and non embryogenic type. Callus formation from embryos and leaf explants could be observed after 6 - 8 d culture. Callus was initiated between the radicle and cotyledon of the embryo, at the position where the basal plate would be formed. Data in Table 1 show that up to 100% of the embryos formed callus and 80% of the embryos formed compact callus. Compared to callus induction on mature embryos, the frequency of callus induction on leaf explants was much lower. A distinct difference in the callus induction response between explants from different positions of the leaf was observed. The upper + middle explants (numbers 2 and 3) exhibited poor callus growth at low frequency (up to 8.3%). The majority of these explants showed only swelling along the cut surfaces and after a number of weeks became necrotic. In contrast, up to 75% of the explants containing the meristem, formed callus. Compact callus formation in leaf explants ranged from 0 to 55%. The compact callus had the same appearance as the compact callus cultures derived from mature embryos.

Table 1. Callus initiation from different explants of *A. ampeloprasum* var. *porrum* L. on MS medium containing 1 mg/l 2,4-D.

Explant	Cultivar	Explants cultured (N)	Explants with callus (%)	Explants with compact callus (%)[a]
Embryo	Porino	225	92	70
	Tilina	225	100	80
Leaf 1	Porino	60	75	55
2	Porino	60	3	1.6
3	Porino	60	1.6	1.6
Leaf 1	Tilina	60	68	22
2	Tilina	60	8.3	1.6
3	Tilina	60	0	0

[a] percentage of explants producing compact callus was assessed after 6 weeks of culture.

Effect of medium composition on callus formation

Results in Table 1 showed that embryos gave a high callus response and therefore embryos of the cultivar 'Tilina' were used for the comparison of different callus initiation media. Statistical analysis showed that no significant effect of the media tested was found with respect to number of explants that produced compact callus. The percentage of explants that formed compact callus was lowest on N_6 medium with 2% sucrose (Table 2). Maximum frequency of explants producing compact callus could be achieved when embryos were cultured on MS medium with 3% sucrose.

Table 2. Effect of callus induction medium on compact callus formation from zygotic embryos of *A. ampeloprasum* var. *porrum* L. cv Tilina.

Callus induction medium[a]	Explants with callus (%)	Explants with compact callus (%)[b]
MS3	100	90
MS2	100	70
BDS3	100	64
BDS2	100	65
$N_6$3	100	80
$N_6$2	100	55

[a] MS3 = MS + 3% sucrose, MS2 = MS + 2% sucrose, BDS3 = BDS + 3% sucrose, BDS2 = BDS + 2% sucrose, $N_6$3 = N_6 + 3% sucrose, $N_6$2 = N_6 + 2% sucrose.

[b] percentage of explants producing compact callus was accessed after 6 weeks of culture.

Effect of different cultivars and accessions on compact callus induction and regeneration

All cultivars produced compact callus, although the percentage of embryos doing so varied considerably (from 21% to 80%; Table 3). The four accessions had the lowest percentage of embryos forming compact callus (21% to 35%). The genotypes, within cultivars and accessions, showed differences for the amount of compact callus that was formed (results not presented).

Table 3. Callus initiation and regeneration from embryo-derived compact callus cultures of *A. ampeloprasum* var. *porrum* L.

Cultivar and accession[a]	Embryos with compact callus (%)[b]	Calli with somatic embryos (%)[c]	Mean no. of somatic embryos per callus[d]	Calli with shoots (%)	Mean no. of shoots per callus[e]
JGPC	63	99 (120)	5.6 (0.51)	94	6.7 (0.70)
Tilina	80	91 (119)	4.0 (0.43)	96	6.6 (0.70)
VVG	70	98 (120)	5.7 (0.51)	94	6.6 (0.70)
Elbeuf	52	97 (96)	5.6 (0.56)	92	6.5 (0.77)
Arcona	66	96 (120)	4.6 (0.46)	88	5.2 (0.62)
Arkansas	65	92 (119)	4.3 (0.45)	84	4.7 (0.59)
90002	35	99 (84)	5.2 (0.57)	92	4.3 (0.67)
Varna	38	91 (110)	3.9 (0.44)	90	3.7 (0.54)
90003	21	46 (54)	3.0 (0.55)	59	3.1 (0.70)
Porino	70	83 (120)	3.0 (0.37)	73	3.0 (0.47)
90004	25	79 (89)	4.0 (0.50)	70	2.8 (0.52)
90001	26	20 (51)	0.5 (0.22)	20	0.3 (0.22)

[a] JGPC = 'Jaune Gros du Poitou Clause', VVG = 'Violet de St. Victor Gautier.
[b] percentage of embryos producing compact callus was assessed after 6 weeks of culture on initiation medium (MS + 1 mg/l 2,4-D)
[c] within brackets the number of calli cultured is given.
[d] mean number of somatic embryos was assessed after 3 weeks of culture on MSK medium, within brackets the standard error is given.
[e] mean number of shoots was assessed after 8 weeks of culture on MSK medium, within brackets the standard error is given.

The calli turned green and showed protuberances within a week after transfer of compact calli to regeneration medium. After a further culture period of 2 weeks, the green areas differentiated into somatic embryos. These showed the characteristic bipolar appearance even during the early stages of development. The somatic embryos usually formed in clusters on the surface of the callus but also appeared singly (Fig. 1B). Detaching the embryos at this early stage inhibited further growth. When leaving them in contact with the callus the somatic embryos subsequently germinated and developed into plantlets (Fig. 1 C,1D). The data presented in Table 3 show that somatic embryos were formed in all cultivars, but at varied frequencies.

There were significant differences in regeneration frequency between the cultivars (Table 3.) Statistical analysis showed that a classification of the cultivars and accessions into three groups accounted for 88% of the variation in shoot regeneration found between the cultivars and accessions. The first group was comprised of the highest regenerative cultivars 'Elbeuf', 'JGPC', 'Tilina' and 'VVG'. Within this group the regeneration frequency was 6.7 shoots per callus. The second group, presenting 7 cultivars, showed a lower regeneration frequency (2.8 to 5.2 shoots per callus). The third group consisted of only one accession (90001) that showed a very low regeneration capacity. On average, only 0.3 shoots were formed per callus and the percentage of responsive calli was low (20%). The accession 90003 could not be classified, because of its heterogeneous nature and the large number of missing values, due to the fact that not all embryos produced enough callus. In most cases, cultivars that formed a high number of somatic embryos also exhibited a high shoot production. However, for the accessions 90004 and 90002 and the cultivar 'Varna' only part of the somatic embryos that were formed after 3 weeks germinated and developed into shoots. On the other hand, the number of somatic embryos formed by the cultivar 'Tilina' was much lower than the number of shoots which eventually formed.

The plantlets developed into phenotypically normal and well-rooted plants that flowered and set seed. As determined by flow cytometric analysis, plants regenerated from young (3 months) and old (10 months) callus cultures had the normal tetraploid DNA content.

Discussion

In leek, mature zygotic embryos are highly responding explants for the initiation of embryogenic callus cultures. The Murashige and Skoog (MS) medium, supplemented with 2,4-D, proved to be a suitable medium for the induction of embryogenic callus on these zygotic embryos. The embryogenic callus cultures were compact and nodular and similar in appearance to those obtained for gramineous species (Vasil 1985) and other *Allium* species (Phillips and Luteyn 1983; Van der Valk *et al.* 1992). The desired callus type was often surrounded by a friable and non-embryogenic callus, thus making continued selection for the compact, regeneration-competent callus type necessary. Mature and immature embryos have been successfully used to initiate embryogenic callus cultures for the major species within the Gramineae (Vasil 1985) and within the genus *Allium* (Phillips and Luteyn 1983; Van der Valk *et al.* 1992).

While for most *Allium* species, regeneration has been reported to occur through organogenesis, in a few species somatic embryogenesis has been reported, i.e., *A. sativum* (Abo El-Nil 1977), *A. cepa* (Dunstan and Short 1978; Phillips and Luteyn 1983; Van der Valk *et al.* 1992), *A. carinatum* (Havel and Novák 1988), *A. fistulosum* (Shahin and Kaneko 1986) and *A. fistulosum* x *A. cepa* (Lu et al. 1989). In this study, plant regeneration appeared to occur mainly through somatic embryogenesis. However, plant regeneration via organogenesis could not be excluded. Due to the fact that on regeneration medium somatic embryos germinated very easily, all calli with embryogenic sectors gave rise to plantlets, independent of the original explant type. The embryogenic calli, which so far have been maintained for about a year are still capable of regenerating plants.

A genotype-dependent response was observed for the

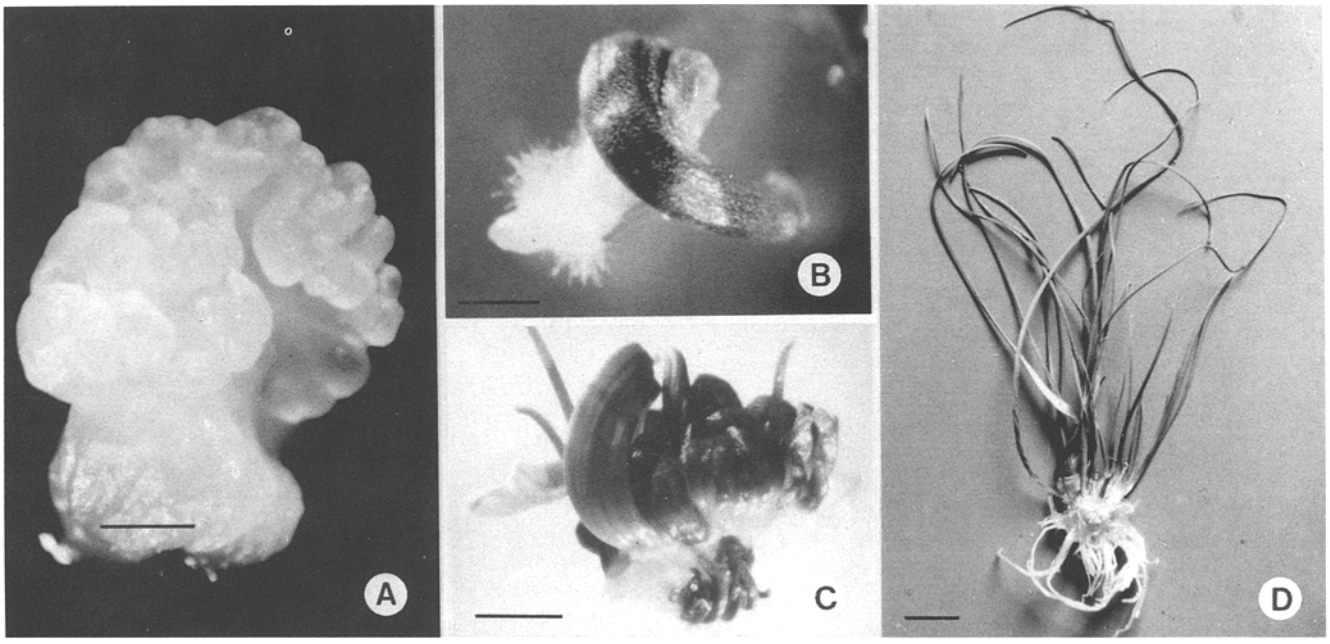

Fig. 1. Somatic embryogenesis and plant regeneration from compact callus cultures of *A. ampeloprasum* var. *porrum* L. [A] Compact and embryogenic callus (Bar = 1 mm). [B] Early stage of a single somatic embryo (Bar = 1 mm). [C] A cluster of germinating somatic embryos attached to callus derived 3 weeks after transfer to MS + 1 mg/l kinetin (Bar = 2 mm). [D] Regenerated plantlets (Bar = 1 cm).

formation of shoots. Variation in shoot regeneration within cultivars from *Allium* callus cultures has also been reported by other authors (Phillips and Hubstenberger 1987; Phillips and Luteyn 1983). It is therefore recommended to choose the cultivars with the highest regeneration capacity and to select within these cultivars for the high, responding genotypes for future experiments.

This study has shown that it is possible to develop a very simple and efficient callus induction and plant regeneration system for leek. Provided that compact calli were cultured, a high frequency of calli with shoots could be obtained for almost all cultivars. The procedure is generally applicable as shown by the fact that we were able to regenerate plants from all cultivars and accessions tested.

The compact and embryogenic callus cultures derived in this study were used to establish a cell suspension. Transfer of the compact and embryogenic callus to liquid medium did not lead to a well-dispersed suspension culture, although the cultures in liquid medium kept the ability to form somatic embryos and shoots for about 6 months. In further experiments we have been able to distinguish new callus types that are morphologically different from those observed in this study. These callus types will be characterized by histological examination and the amenability for the establishment of cell suspension cultures and the subsequent isolation and culture of protoplast will be tested.

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