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Culture of Protoplasts Isolated from Leaves and Callus Cultures of *Allium sativum* and *Allium longicuspis*: a Preliminary Report

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5 Figures, 4 Tables

Zusammenfassung

Basalteile von Blättern junger Pflanzen von *Allium sativum* L. und *Allium longicuspis* Regel. und Kalluskulturen aus diesen wurden verglichen als Ausgangsmaterial zur Isolation und Kultur von Protoplasten. Der Anteil von Protoplasten, ihr Überleben in Kultur und ihr Vermögen zur Zellwand-Regeneration und Zellteilung wurden für beide Genotypen in Abhängigkeit der enzymatischen Behandlungsdauer einerseits und der Zusammensetzung des Kulturmediums andererseits untersucht. Die aus den Blättern von *A. sativum* und *A. longicuspis* isolierten Protoplasten zeigten die erste Zellteilung nach 5–6 Tagen in einem Gentamycin als Antibiotikum enthaltenden Medium, und die Protoplastenkulturen überlebten höchstens 29–34 Tage. Kallus-Protoplasten von *A. sativum* im selben Medium, aber ohne Antibiotikum, zeigten die ersten Zellteilungen nach 2–3 Tagen. Unter denselben Kulturbedingungen setzte die Zellteilung bei Kultur aus *A. longicuspis* im Verlauf von 2 oder 3 Tagen ein, reproduzierbar aber in sehr niedriger Häufigkeit; nur selten wurden kleine Zellkolonien nach 20 Tagen Kultur beobachtet. Kallus-Protoplasten von *A. sativum* und *A. longicuspis* überlebten 40–50 Tage in Kultur.

***Allium longicuspis* – *Allium sativum* – Kallus-Kultur – Kontamination – Gentamycin – Protoplasten-Kultur**

Abstract

Basal parts of leaves of young plants of *Allium sativum* L. and *Allium longicuspis* Regel. and callus cultures derived from them were compared as starting materials for the isolation and culture of protoplasts. The yield of protoplasts, their survival in culture, as well as their capacities for cell wall regeneration and cell division induction were studied for both genotypes as a function of the enzymatic treatment duration on the one hand and the composition of culture media on the other hand. The protoplasts isolated from leaves of *A. sativum* and *A. longicuspis* exhibited the first cell division after 5 to 6 days in a medium containing gentamycin

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as an antibiotic and the protoplast cultures survived 29 to 34 days longest. Callus protoplasts from *A. sativum* cultured in the same medium, except for the antibioticum, showed the first cell divisions after 2 to 3 days of culture. Under these same culture conditions, cell cultures derived from protoplasts of *A. longicuspis* were dividing at the course of 2 or 3 days reproducibly though in very low frequency and the formation of small cell colonies was observed, though infrequently, after 20 days culture. Callus protoplasts of *A sativum* as well as *A. longicuspis* survived 40 to 50 days in the culture.

***Allium longicuspis* – *Allium sativum* – callus culture – contamination – gentamycin – protoplast culture**

Introduction

Garlic *(A. sativum)* is generally a pollen-sterile plant and thus does not form seeds, except for a very few wild species (ETOH, 1986). A viral phenomenon (mycoplasma phenomenon) has been postulated to cause a disease of the tapetum in garlic (KONVIČKA, 1972, 1973). In this connection, contamination of garlic explants *in vitro* was recently mentioned by RAUBER and GRUNEWALDT (1988) as well as chronic contamination by viruses of garlic plants (BOS, 1983, and references therein). These points raise the question as to the influence of such infections on the flower biology resulting in garlic sterility, but also on the physiological characteristics of *in vitro* cultures. Thus, propagation of garlic is exclusively possible in a vegetative way and the absence of sexual reproduction impoverishes its genetic variablility very much. Nevertheless, garlic plays an important role in medicine due to its cytotoxic, antibacterial, antitumor or else antithrombic effects on animal cells (FUJIWARA and NATATA, 1967) and it would be interesting to be able to conduct the production of garlic crops. For that reason, garlic regeneration from protoplasts is a goal for the next future. This step is a prerequisite to overcome the problems involved in sexual reproduction of *Allium* plants using somatic, gametosomatic hybridization or for direct transformation of protoplasts.
The aim of this work was to compare the quality of protoplast suspensions isolated from different materials that originated from *A. sativum* and *A. longicuspis* and the growth characteristics of the cell cultures derived from these protoplasts as a function of the isolation process and the culture conditions. Hypotheses for explaining the blockade of the division and supporting future work are presented.

Materials and Methods

Plant material

Two species of garlic *Allium sativum* L. cv. "Moravan" and *Allium longicuspis* Regel. wild type (collected in area of Issyk-Kul lake in Uzbekistan) from the germplasm collection of the Research Institute of Vegetable Growing and Breeding in Olomouc were used for the experiments. *A. sativum* is a pollen sterile plant and *A. longicuspis* has fertile pollen, but sterile ovules.
Virus free plants, originally obtained from meristem-tip cultures (HAVRÁNEK, 1972), were grown on an artificial substrate ("Perlit") from cloves in a greenhouse, under natural light conditions and at 23–27 °C. Young plantlets 10 to 20 cm high were harvested and washed in water. Basal parts of leaves were either used directly for protoplast isolation or for induction of calli, which were used as source material for protoplast isolation. Roots of both *A. sativum* and *A. longicuspis* were also used for callus induction. Apical parts of roots and light green basal parts of leaves were rinsed in 70 % ethanol and were

surface sterilized for 25 min in 5 % chloramin B (Natrium benzensulfochloramidicum, 1.5 % of active chlorine plus released oxygen). Then they were washed in 70 % ethanol and rinsed five times in sterile bidistilled water. The sterilization procedure was the same for explants intended for protoplast isolation or for callus induction.

Isolation and culture of leaf protoplast

Surface sterilized leaves were cut into pieces of 2 to 3 mm and placed in 100 mm Petri dishes with the enzymatic solution (1 g FW/10 ml) containing 0.8 % (w/v) cellulase "Onozuka R-10" (Serva, Germany), 0.2 % (w/v) "Macerozyme R-10" (Serva) and 0.9 % (w/v) $CaCl_2 \cdot 2H_2O$, pH 5.7. The osmolality of the digestion solution was adjusted to 560–600 mOs/kg H_2O with mannitol. The enzyme solution was sterilized by filtration through 0.2 μm filter (Sartorius, Germany). Leaves were digested for 16 to 22 hours depending the rapidity of the digestion, in dark, at 25 °C, and without shaking.

The protoplast suspension was strained through a 42 or 57 μm nylon mesh filter according to the size of the protoplasts and centrifuged at 70 × g for 5 min. Pellet was resuspended in a washing medium (WM) (pH 5.6) containing 0.4 M glucose and 18 mM $CaCl_2 \cdot 2H_2O$ and recentrifuged at 60 × g for 5 min. This washing procedure was repeated three times, but the last centrifugation was done in the culture medium. The pellet of protoplasts was resuspended in a culture medium at a density of 5×10^5 protoplasts per ml.

Protoplasts were cultured in three different culture media (Table 1) with and without the antibiotic gentamycin (25 mg/l) (Pharmachim, Bulgaria) known to affect protein synthesis in a wide range of gram-positive and gram-negative bacteria. Protoplasts were cultured in the dark, at 25 °C in 60 mm Petri dishes. The viability of protoplasts was determined by FDA (fluorescein diacetate) staining (WIDHOLM, 1972) and cell wall regenerations by calcofluor white staining (NAGATA and TAKEBE, 1970).

Callus induction

Surface sterilized leaf basal parts of plants were cut first into discs of 2 to 3 mm diameter and then quartered and placed singly onto the induction culture medium solidified (0.8 % agar, Difco, USA) in 20 cm tubes. Apical parts of roots (20 mm length) were cut into pieces 2 to 3 mm long and placed on culture medium. Every explant was cultured at 25 °C, with an illumination of 3,200 to 3,600 lx with 16-hour photoperiod. For callus induction three different culture media differing mainly as to their hormone con-

Tab. 1. Composition of media used for protoplast culture. All media contained 2 % (w/v) sucrose. Osmolality of all media was adjusted with mannitol to 560–600 mOs/kg H_2O for leaf protoplasts and to 580–660 mOs/kg H_2O for callus protoplasts and the pH was adjusted to 5.7 before autoclaving

Signature of medium	Basal salts	Growth regulators	References
MSW	MS[a])	9.1 μM 2,4-D[c]) 2.2 μM BAP	WANG et al., 1986
BDSW	BDS[b])	9.1 μM 2,4-D[c]) 2.2 μM BAP	–
H7	BDS[b])	1.0 μM 2,4-D 5.0 μM kinetin	DOLEŽEL (unpublished)

[a]) Murashige and Skoog medium (MURASHIGE and SKOOG, 1962)
[b]) Dunstan and Short medium (DUNSTAN and SHORT, 1977)
[c]) Hormone concentrations (μM) are calculated from Material and Methods in WANG et al. (1986)

Tab. 2. Composition of media used for callus induction. All media contained 2 % (w/v) sucrose and their pH was adjusted to 5.7 before autoclaving. For MS-1, 2, 4-D concentration was half of the one used by HAVRÁNEK and NOVÁK (1973)

Signature of medium	Basal salts	Growth regulators	References
MS-1	MS[a])	10 μM kinetin 10 μM IAA 2.5 μM 2, 4-D	HAVRÁNEK and NOVÁK 1973 (modified)
BDS-2	BDS[b])	8.9 μM BAP[c])	PHILLIPS and LUTEYN, 1983
BDS-3	BDS[b])	10 μM kinetin 10 μM IAA 5 μM 2, 4-D	NOVÁK et al., 1982

[a, b]) See table 1
[c]) Hormone concentration (μM) is calculated from Material and Methods in PHILLIPS and LUTEYN (1983)

tent were used (Table 2). For growth, induced calli 2 to 3 mm big were maintained in the dark at 25 °C on medium H7 solidified by agar (0.8 %) and free of mannitol and transferred on fresh medium every month.

Isolation and culture of callus protoplasts

Calli, one week after a transfer on fresh medium, were cut off in small pieces and placed into 100 mm Petri dishes containing the enzyme solution (per 5 g FW/10 ml), Enzyme solution contained 2 % (w/v) cellulase "Onozuka R-10", 0.2 % (w/v) "Macerozyme R-10", 1 % (v/v) pectinase (Sigma, USA), 0.9 % (w/v) $CaCl_2 \cdot 2\,H_2O$, pH 5.6 (KIM et al., 1986, modified). The osmolality was adjusted to 600–690 mOs/kg H_2O with mannitol. The enzyme solution was sterilized by filtration through 0.2 μm filter. Digestion was studied as a function of time for 6 to 23 hours in the dark, at 25 °C and without shaking.
Protoplast suspension was strained through a 42 or 72 μm mesh nylon filter and centrifuged at 70 × g for 5 min. Protoplasts were washed once according to the same procedure as described for leaf protoplasts. But in the case of protoplasts isolated from calli, a further purification by density gradient was performed. Protoplasts (2 ml) were partitioned by a centrifugation at 60 × g for 15–20 min between a two phase mixture containing 20 % (w/v) sucrose (3 ml) and WM (3 ml). Most of the debris pelleted and purified protoplasts were layered at the interface glucose/sucrose. They were collected and washed once more in WM by a centrifugation at 50 × g for 6 min. Final pellet was resuspended in a culture medium at the density of 5×10^5 protoplasts per ml. Protoplast were cultured in the dark at 25 °C in medium H7 (Table 1).

Results

Behaviour of leaf protoplasts under conditions of *in vitro* culture

Suspensions of fresh protoplasts isolated from both genotypes were heterogeneous as to the size and the structure of the protoplasts, as shown in Figure 1 a for *A. sativum* and 1 b for *A. longicuspis*. According to our procedure the yields of protoplasts isolated from basal leaf

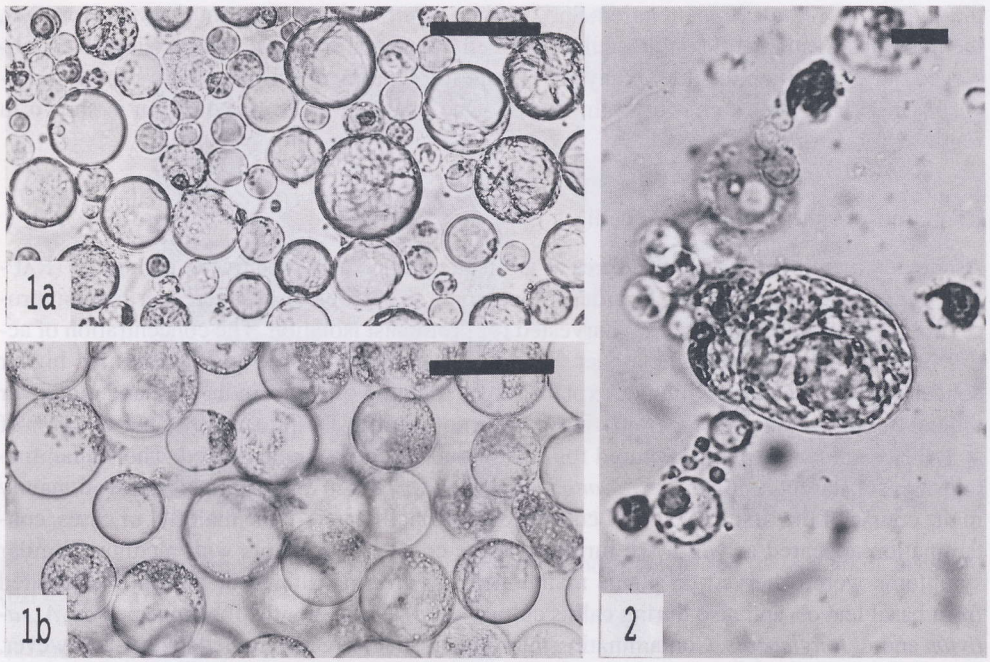

Fig. 1. Freshly isolated leaf protoplasts of *A. sativum* (a) and *A. longicuspis* (b) (17 hours of digestion) (bar = 100 μm)

Fig. 2. First cell division of *A. sativum* leaf protoplasts after 6 days of culture in H7 medium (bar = 100 μm)

parts of *A. sativum* and *A. longicuspis* were similar, $1.57 \times 10^6 \pm 0.15$ protoplasts/lg FW ($\times \pm$ s.e., n = 21 independent experiments) and $1.38 \times 10^6 \pm 0.10$ protoplasts/lg (n = 21 independent experiments), respectively. In these experiments the viability of protoplasts at the beginning of culture was for *A. sativum* 75.4 % ± 2.3 and for *A. longicuspis* 74.0 % ± 2.7. Commonly, the digestion time for an optimal yield of protoplasts was 18 hours for both *A. sativum* and *A. longicuspis*.

The protoplasts isolated from the two species were cultured in three different media (Table 1) either without gentamycin or with gentamycin. Protoplasts cultured without gentamycin, whatever the medium was, survived for a very short time, because of chronic contamination by unknown organisms occurring 2 to 3 days after the beginning of the culture. Thus, viability, cell wall regeneration, and cell division were studied as a function of time only in the case of protoplasts cultured in the presence of the antibiotic. The outburst of contamination was repressed by adding gentamycin to final concentration of 25 mg/l to the protoplast culture without reduction of viability during the first day of culture and the protoplasts survived in the culture as opposed to protoplasts cultured in the absence of the antibiotic. Viability of protoplasts was decreased with culture time step by step down to zero after 29 days for *A. sativum* and after 34 days for *A. longicuspis*. Cell wall regeneration was observed after 40 hours of culture for *A. sativum* and after 25 hours for *A. longicuspis*. At the same time the budding of protoplasts was observed. Significant influences of the culture media were not observed on proto-

plast viability, cell wall regeneration, and budding of protoplasts (data not shown). However, the main observation is that almost all of the cells were not dividing; cell divisions were observed quite exceptionally after 6 days of culture for *A. sativum* and after 5 days for *A. longicuspis* in H7 medium only, for both species. One example of such a division is shown in Figure 2 for *A. sativum*.

Conditions for the production of calli

As already mentioned, *in vitro* cultures of garlic are particularly sensitive to pathogens (RAUBER and GRUNEWALDT, 1988). In order to overcome that problem, a severe sterilization procedure was applied to the material harvested for protoplast isolation. The concentration of active chlorine (1.5 %) was the upper limit for maintenance of explant viability; higher concentrations led to the killing of explants, to a reduction of callus induction, and moreover did not turn out to have a bigger efficiency (data not shown). Despite the presence of 1.5 % of active clorine, leaf explants cultured for cally induction were contaminated. The contamination was substantially lower for *A. longicuspis* than for *A. sativum* and was observed mainly in the course of the first five days of culture, exceptionally later. In the majority of cases, contamination appeared as yellowish turbidity at the contact of explants with medium. Similar symptoms were observed on plants maintained *in vitro*, in cultures of protoplasts isolated from basal leaves, and also during callus culture over several months for both species, *A. sativum* and *A. longicuspis*. Contaminating microorganisms were not studied in detail; however, preliminary tests indicated the presence of about six gram-positive and gram-negative bacterial species in the cultures. As to root explants, contamination was very low and no differences appeared between the two genotypes. Contrary to leaves, different kinds of contamination were observed according to visual criteria. As a control, the totally fertile genotype, *A. porrum* cv. "Elephant", was tested under the same conditions as the ones used for *A. sativum* and *A. longicuspis*. As expected, contamination of leaf and root explants was negligible (about 0.5 %) and callus induction was high (about 70 %).
The efficiency of the three media for callus formation from leaf explants was tested over 40, 80 and 120 days of culture (Table 3). In order to avoid a wrong estimation of callus induction,

Tab. 3. Callus induction from leaf explants of *A. sativum* and *A. longicuspis*

Species	Culture media[a])	Number of noncontamined explants	Percent of responsive explants[b]) after:		
			40 days	80 days	120 days
A. sativum	MS-1	116	43	71	78
	BDS-3	49	12	14	16
	BDS-2	30	0	0	0
A. longicuspis	MS-1	198	38	88	88
	BDS-3	168	31	42	47
	BDS-2	161	0	0	0

[a]) See table 2
[b]) Calculated from non-contaminated explants

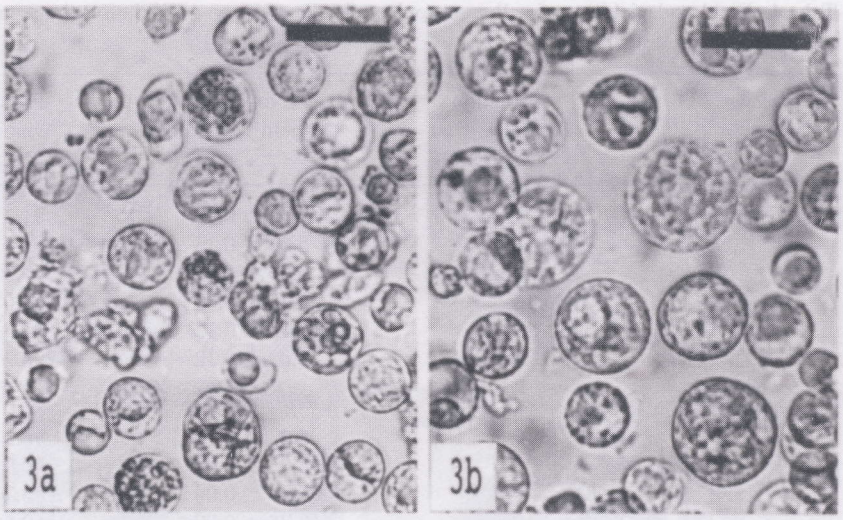

Fig. 3. Fresh callus protoplasts isolated from *A. sativum* (a) and *A. longicuspis* (b) (8 hours of digestion) (bar = 100 μm)

it has been expressed in % of responsive explants from non-contaminated explants. The highest number of calli was produced from leaf explants cultured on MS-1 medium for both *A. sativum* and *A. longicuspis*. Contrary to this, no calli at all were produced from both species in BDS-2 medium, a medium which was successfully used for callus induction of *A. cepa* (PHILLIPS and LUTEYN, 1983). BDS-3 medium was of intermedian efficiency for callus production for both species. Calli production started after three weeks of culture and extended over 8 to 10 weeks. Just a few percent of calli appeared later on. As to root explants from both *A. sativum* and *A. longicuspis* they turned green in the three media and exhibited only a slight enhancement of their size but never produced calli.

Calli produced from leaf explants were able to form shoots in H7 medium at 25 °C and with 16 hours photoperiod. After transfer of the neoformed shoots onto BDS medium in the absence of growth regulators, root formation was always observed and plantlets, progressively transferred into soil, grew without problems.

Behaviour of callus protoplasts under conditions of *in vitro* culture

The calli derived only from the basal parts of leaves of *A. sativum* and *A. longicuspis* were used as source material for isolation of protoplasts after their multiplication on H7 agar medium. The yield of protoplasts and their viability were studied as a function of the length of enzymatic treatment, namely from 6 to 23 hours. Fresh protoplasts isolated after 8 hours of digestion are shown on Figure 3a for *A. sativum* and Figure 3b for *A. longicuspis*. The two suspensions appeared to be as heterogeneous as the ones isolated from leaves (Fig. 1a, b). Maximum yield of protoplasts was observed after 19 hours of enzymatic treatment for *A. longicuspis* and after 16 hours for *A. sativum* (Table 4). However, the viability of protoplasts was highly decreased after such a long period as compared to maximum viability determined for protoplasts of both *A. sativum* and *A. longicuspis* after 7 and 8 hours of iso-

Tab. 4. Yield and viability of callus protoplasts in dependence on isolation time (means of 13–21 independent experiments)

Species	Isolation time (in hours)	Protoplast yield[a]) ($\times 10^6$) $\times \pm$ s.e.	Protoplast viability (in %) $\times \pm$ s.e.
A. sativum	7	4.34 ± 0.10	91.2 ± 1.5
	8	5.64 ± 0.13	91.7 ± 1.2
	9	6.04 ± 0.11	80.5 ± 1.3
	16	6.45 ± 0.09	72.0 ± 1.8
	19	6.25 ± 0.12	65.3 ± 1.7
	23	6.03 ± 0.12	58.2 ± 1.5
A. longicuspis	6	3.67 ± 0.10	84.0 ± 2.0
	7	5.23 ± 0.10	96.2 ± 1.3
	8	5.75 ± 0.08	90.9 ± 1.6
	9	6.95 ± 0.06	86.7 ± 1.7
	19	7.75 ± 0.09	69.6 ± 2.1
	23	7.65 ± 0.09	61.3 ± 2.1

[a]) Yield of protoplasts is referred to 1 g of callus

lation (Table 4). The viability and yield of callus protoplasts were higher when compared to protoplasts isolated from fresh leaves of *A. sativum* and also *A. longicuspis*.

These observations indicated that the period of 7–8 hours for digestion could be critical under our conditions for viability and culture of protoplasts. Thus, protoplast cultures were initiated from protoplast suspensions after 7–8 hours digestion or else 19 hours as an extreme condition of digestion. The protoplasts were cultured in liquid culture medium H7 without gentamycin as callus protoplast cultures were almost never contaminated. A long enzymatic treatment of 19 hours decreased by about 25 % the viability of fresh protoplasts for both genotypes, *A sativum* and *A. longicuspis*. However, during the culture of the protoplast-derived cells, viability was decreased to the same proportion without respect to the digestion procedure used, except for *A. longicuspis*, which shows only a slight decrease in viability until 5 days. Cell wall formation was observed for both, *A. sativum* and *A. longicuspis*, after 24 hours, but more frequently after 35 hours of culture, for protoplasts isolated after short as well as long enzymatic treatment. Handicapped budding was always observed.

By comparison with cultures of leaf protoplasts, cell division was obtained more easily and more frequently when cultures were initiated from callus protoplasts. For *A. sativum*, first cell divisions were observed after 2 to 3 days of culture for protoplasts obtained by short as well as long enzymatic treatment (Fig. 4), but it remains a rather rare event and moreover second cell divisions and colony formation were not observed. Nevertheless, callus protoplasts from *A. longicuspis* were more responsive and it has been possible to measure the frequency of occurrence of cell division for that genotype. First induction of cell divisions was not dependent on the duration of the enzymatic treatment for *A. longicuspis* and was observed in the course of 2 to 3 days of culture (Fig. 5 a). The frequency of cell division (unequal type largely) was very low (0.1 %). Less frequently but significantly second cell division (Fig. 5 b) was obser-

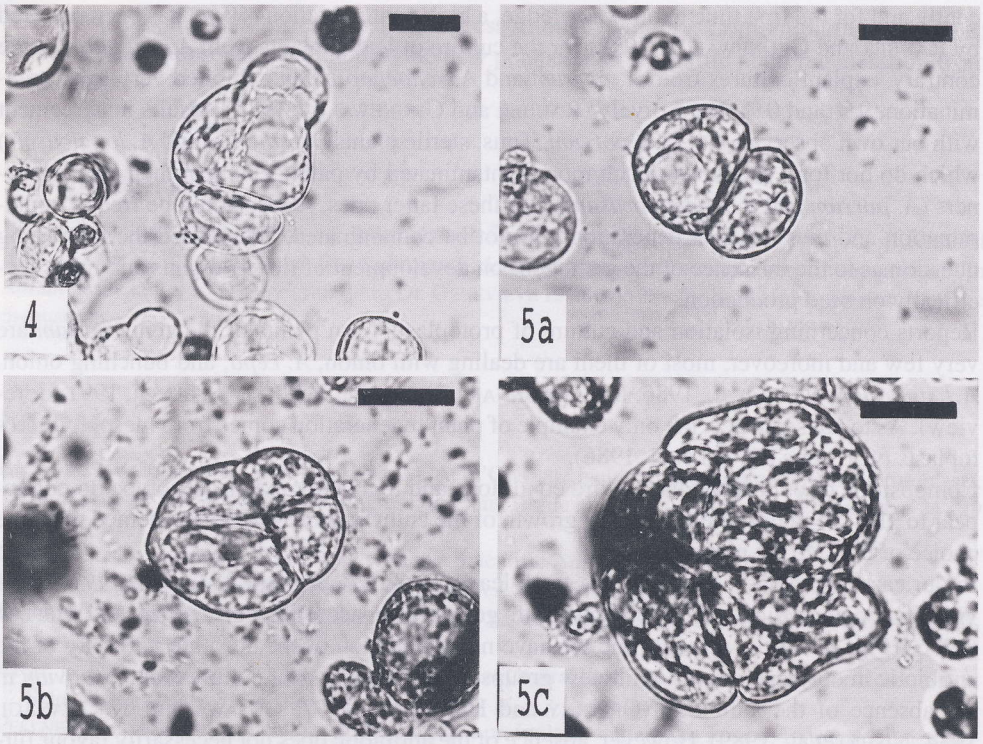

Fig. 4. Culture of protoplasts from callus of *A. sativum* (8 hours of digestion) after 3 days of culture in H7 medium (bar = 100 μm)

Fig. 5. Culture of protoplasts from callus of *A. longicuspis* (8 hours of digestion) after 2 days (a), 12 days (b), and 20 days (c) of culture in H7 medium (bar = 100 μm)

ved after 7 to 12 days for protoplasts obtained by short enzymatic treatment and even small cell colonies were observed (Fig. 5 c) after 20 days of culture. Protoplast-derived cells of both, *A. sativum* and *A. longicuspis*, survived in good health in H7 medium for periods as long as 40–50 days.

Discussion

Protoplast cultures of garlic have as yet not been really successful. Two source materials for protoplast isolation and culture were compared, fresh leaves and calli originated from leaf explants. Root explants did not produce calli and will not be taken into consideration in future work.

Previous works in our laboratory (HAVRÁNEK and NOVÁK, 1973; NOVÁK et al., 1982) allowed to develop calli production from *A. sativum*. We got easily calli from leaf explants with the highest yield using the medium of HAVRÁNEK and NOVÁK (HAVRÁNEK and NOVÁK, 1973), partly modified, but we observed a high level of contamination (78 %), a problem which was not mentioned in the reports of HAVRÁNEK und NOVÁK (1973) and NOVÁK et al. (1982). Re-

cently and for the first time to our knowledge, a high contamination (60 %) has been reported by RAUBER and GRUNEWALDT (1988) for the culture of leaf explants from *A. sativum*. On the contrary, explant cultures from *A. porrum* and *A. schoenoprasum* exhibited very low contamination, 9 % and 0 %, respectively (RAUBER and GRUNEWALDT, 1988), results in agreement with our own observations on *A. porrum*. Thus, sterile plants (*A. sativum* and *A. longicuspis*) which do not form seeds, are much more contaminated by pathogens than their fertile partners (*A. porrum* and *A schoenoprasum*). For these latter ones, seeds should be free of contamination and thus the next generation will not be contaminated. This raised the interesting question as to the influence of the pathogens on development of flower organs and more specifically on seed production.

Reports concerning isolation and culture of protoplasts from plants of the genus *Allium* are very few and moreover, most of them are dealing with onion, *A. cepa*, and bunching onion, *A. fistulosum* (WANG et al., 1986; BALAKRISHNAMURTHY et al., 1990; see FELLNER, 1991 for review). As to consequence, the only example of plant regeneration comes from mesophyll protoplasts of *A. cepa* (WANG et al. 1986).

Composition of culture media, culture conditions and to a lesser extent, genotype do not appear to play the major role for getting growth of the culture; more important seemed to be the choice of the starting material.

In our case, protoplasts isolated from fresh leaves of *A. sativum* and *A. longicuspis* only divide if contamination was overcome with gentamycin according to ZEIGER and HEPLER (1976) (Fig. 2). At the present time we have no explanation of the absolute necessity of the antibiotic in our experiments while few groups reported successful cultures for *A. sativum* in the absence of the antibiotic (OPATRNÝ and HAVRÁNEK, 1977; OOSAWA and TAKAYANAGI, 1984; NISHIO et al., 1989). However, absence of the antibiotic does not necessarily favour further divisions, as among these three reports only OOSAWA and TAKAYANAGI (1984) observed several-fold cell divisions.

The best source material for protoplast isolation and culture was the calli produced from leaf explants. Protoplast cultures from *A. sativum* and *A. longicuspis* did not display real differences of viability and cell division in dependence on the digestion time used (Table 4). However, colony production appeared only in *A. longicuspis* cultures and when protoplasts were isolated after short time digestion (Fig. 5 c).

To our knowledge, this is the first attempt that calli of garlic plants were used for initiating protoplast cultures. A first advantage of this material was the absence of contamination of the protoplast culture. More important was the fact that we got reproducible formation of small colonies when starting from calli derived from leaf explants of *A. longicuspis*. Difficulties with respect to further growth could derive from heterogeneity of the population in terms of ploidy, as has been demonstrated by HAVRÁNEK and NOVÁK (1973) and NOVÁK et al. (1982) who showed that *A. sativum* calli revealed a mixoploid character. Moreover, the blockade of the cultures could be due to the accumulation of autotoxic sulfur compounds, as alliin (or allicin and ajoene) (BLOCK, 1985) or flavour precursors produced in garlic plants and also in calli of garlic (LANCASTER et al., 1988). This problem would be probably much more pronounced in cultures of protoplasts isolated directly from leaves or from differentiating cultures than for protoplasts isolated from undifferentiated callus cultures, in which the content of alliin is two times lower than in differentiating cultures (MALPATHAK and DAVID, 1986). Presence of flavour precursors in undifferentiated white calli differed from the representation of flavour precursors in intact garlic plants (LANCASTER et al., 1988). These toxic products can be "neutralized" by SH-compounds such as like cystein, β-mercaptoethanol, glutation or DTT (K. G. WAGNER, pers. comm.). Experiments aiming to eliminate such an eventual toxic

effect in protoplast cultures are under progress in our laboratory. Combining the benefits of these different approaches could provide new perspectives for garlic plant regeneration from protoplasts.

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References

BALAKRISHNAMURTHY, G., G. C. PHILLIPS, M. A. O'CONNEL: Isolation and culture of *Allium* protoplasts. In: Abstracts 7th International Congress on Plant Tissue and Cell Culture. Amsterdam June 24–29 (1990) 7.

BLOCK, E.: The chemistry of garlic and onions. Scientific American 252 (1985) 114–119.

BOS, L.: Viruses and virus diseases of *Allium* species. Acta Horticulturae 127 (1983) 11–29.

DUNSTAN, D. I., K. C. SHORT: Improved growth of tissue cultures of the onion, *Allium cepa*. Physiologia Plantarum 41 (1977) 70–72.

ETOH, T.: Fertility of garlic clones collected in Soviet Central Asia. J. Japanese Society for Horticultural Sciences 55 (1986) 312–319.

FELLNER, M.: Isolation and culture of *Allium* plant protoplasts – literature review. External studies thesis. Charles University. Prague (1991) (in Czech).

FUJIWARA, M., T. NATATA: Induction of tumor immunity with tumor cells treated with extract garlic *(Allium sativum)*. Nature 216 (1967) 83–84.

HAVRÁNEK, P.: Virusfree clones of the common garlic obtained by meristem-tip cultures. Ochrana rostlin 8 (1972) 291–298.

HAVRÁNEK, P., F. J. NOVÁK: The bud formation in the callus cultures of *Allium sativum* L. Zschr. Pflanzenphysiologie 68 (1973) 308–318.

KIM, H. I., Y. H. KIM, T. Y. CHUNG: Studies on protoplasts fusion of *Allium sativum* with *Allium monanthum*. 1. Effects of polyethylene glycol, high pH/Ca^{2+} solution and temperature treatments. Korean Plant Tissue Culture (Korea R.) 13 (1986) 37–44.

KONVIČKA, O.: Cytotaxonomische Studien von vier sterilen Arten der Gattung *Allium*. Biologia Plantarum (Praha) 14 (1972) 62–70.

KONVIČKA, O.: Die Ursachen der Sterilität von *Allium sativum* L. Biologia Plantarum (Praha) 15 (1973) 144–149.

LANCASTER, J. E., E. M. DOMMISSE, M. L. SHAW: Production of flavour precursors [S-alk(en)yl-L-cysteine sulphoxides] in photomixotrophic callus of garlic. Phytochemistry 27 (1988) 2123–2124.

MALPATHAK, N. P., S. B. DAVID: Flavor formation in tissue cultures of garlic (*Allium sativum* L.). Plant Cell Reports 5 (1986) 446–447.

MURASHIGE, T., F. SKOOG: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15 (1962) 473–497.

NAGATA, T., I. TAKEBE: Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta (Berlin) 92 (1970) 301–308.

NISHIO, T., Y. SAKATA, H. YAMAGISHI, Y. TABEI, T. SATO, K. TAKAYANAGI: Protoplast culture in vegetable crops. Development and improvement of culture procedure. Bulletin of the National Research Institiute of Vegetables, Ornamental Plants and Tea A 3 (1989) 67–69.

NOVÁK, F. J., L. HAVEL, J. DOLEŽEL: *In vitro* breeding system of *Allium*. In: FUJIWARA, A. (ed.) Proc. of the 5th International Congress on Plant Tissue and Cell Culture. Tokyo (1982) 767–768.

Oosawa, K., K. Takayanagi: Protoplast approach in vegetable breeding. I. Isolation and culture conditions of protoplasts from mesophyll cells of vegetable crops. Bulletin of the Vegetable and Ornamental Crops Research Station, Series A, No. 12 (1984) 9–28.

Opatrný, Z., P. Havránek: Isolation, cultivation and fusion of protoplasts of common garlic *(Allium sativum)* and leek *(Allium porrum)*. In: Proc. of the International Symposium. Prague (1977) 471–476.

Phillips, G. C., K. J. Luteyn: Effect of picloram and other auxins on onion tissue cultures. J. American Society for Horticultural Sciences 108 (1993) 948–953.

Rauber, M., J. Grunewaldt: *In vitro* regeneration in *Allium* species. Plant Cell Reports 7 (1988) 426–429.

Wang, G. Y., Z. A. Xia, L. F. Wang: Regenerated plantlets from cultured mesophyll protoplasts of onion (*A. cepa* L.). Acta Biologiae Experimentalis Sinica 19 (1986) 409–413.

Widholm, J. M.: The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technology 47 (1972) 189–194.

Zeiger, E., P. K. Hepler: Production of guard cell protoplasts from onion and tobacco. Plant Physiology 58 (1976) 492–498.

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