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Problems of garlic protoplast cultures-literature review, speculation and hypoteses

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FEATURE ARTICLE

PROBLEMS OF GARLIC PROTOPLAST CULTURES - LITERATURE REVIEW, SPECULATIONS AND HYPOTHESES

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1. Introduction

Garlic *(Allium sativum)* is generally a pollen sterile plant and thus does not form seeds, except for a very few wild species (Etoh, 1986). A mycoplasma infection has been postulated to cause a disease of the tapetum in garlic (Konvička, 1972, 1973). In connection, contamination of garlic explants *in vitro* was recently mentioned (Rauber and Graunewaldt, 1988) as well as chronic contamination by viruses of garlic plants (Bos, 1983, and references therein). These points raise the question of the influence of such infections on flower biology ending with 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 very much impoverishes its genetic variability. Nevertheless, garlic plays an important role in medicine in many respects - such as in cytotoxic, antimicrobial, antitumor and/or antithrombic effects on animal cells (Fujiwara and Natata, 1967) and it would be interesting to be able to manage the production of garlic crops. For that reason, garlic regeneration from protoplasts is a goal for future experiments. This step is a prerequisite for getting over the problems involved in sexual reproduction of *Allium* plants using somatic, gametosomatic hybridization or for direct transformation of protoplasts.

Reports concerning the culture of protoplasts from plants of genus *Allium* are very few and moreover most of them deal with onion *A. cepa* and *A. fistulosum* (Wang et al., 1986; Balakrishnamurthy et al., 1990; Fellner, 1991, for detailed review). Consequently, the only example of plant regeneration comes from mesophyll protoplasts of *A. cepa* (Wang et al., 1986). Protoplast cultures of garlic *A. sativum* have not been really successful in the meantime. For that reason, culture of garlic protoplasts is still not very attractive for commercial use, and that is why detailed studies on the behaviour of protoplasts and physiological aspects in the culture have not yet been published.

2. Plant material

Two species of garlic *Allium sativum* L. of different cultivars and *Allium longicuspis* Regel. (wild type - progenitor of *A. sativum,* collected in area of Issyk-kul lake in Uzbekistan) were used for experiments. *A. sativum* is a pollen sterile plant and *A. longicuspis* encloses fertile pollen, but sterile ovules.

For the collection of tissues and for protoplast isolation garlic plants grown in the field or in a greenhouse on soil or artificial substrate were always used. In our experiments (Fellner and Havránek, 1993) in addition, virus-free plants originally obtained from meristem-tip cultures (Havránek, 1972) were used. As a source of protoplasts, surface sterilized garlic leaves were used. In one case garlic cloves, and calli, derived from basal parts of leaves, were also used (Table 1).

3. Culture of protoplasts

3.1. Leaf protoplasts

Leaf material for protoplasts isolation was surface sterilized in different commercial products containing active chlorine plus released oxygen.

At the start of the culture, the protoplast viability was about 75% (determined by Fluorescein diacetate staining) (Widholm, 1972) for *A. sativum* and *A. longicuspis* (Fellner and Havránek, 1993). As for their size and structure, fresh protoplast suspensions, derived from mesophyll cells, were generally much more homogeneous as compared with protoplasts obtained from white basal parts of leaves.

Cultures of protoplasts were realized largely in liquid culture medium of several basic types, complemented by auxins and cytokinins in different concentrations (Table 1). Regeneration of new cell wall (determined by Calcofluor White staining) (Nagata and Takebe, 1970) of cultured protoplasts of *A. sativum* was observed in all published experiments and it took from 40 hours to several days (Opatrný and Havránek, 1977; Fogher and Corti, 1982; Oosawa and Takayanagi, 1984; Havel and Novák, unpublished; Nishio et al., 1989; Fellner and Havránek, 1993). Protoplasts of *A. longicuspis* regenerated the cell wall substantially sooner, over a 25 hour period (Fellner and Havránek, 1993). At the same time, the budding of protoplasts was observed as a matter of course (Opatrný and Havránek, 1977; Fellner and Havránek, 1993). Protoplasts of *A. sativum* survived in the culture only from 2 to 5 weeks and the first cell division (Opatrný and Havránek, 1977) or several-fold cell division (Oosawa and Takayanakgi, 1984) was observed. Efforts to evoke the next development of cells have not been successfull yet. In our case, protoplasts isolated from leaves of *A. sativum* and *A. longicuspis* were cultured either without or with antibiotic gentamicin, whatever the medium, survived a very short time, because of a chronic contamination by unknown organisms occurring 2 to 3 days after the beginning of the culture. Adding gentamicin to the final concentration of 25 mg/l to the protoplast culture, repressed the outburst of contamination. Gentamicin is known to affect protein synthesis in a wide range of gram-positive and gram-negative bacteria. The preliminary determination of the contaminating organisms in the protoplast cultures showed a presence of nonphytopathogenic gram-positive as well as gram-negative bacteria (Fellner, unpublished), and at the same time, gentamicin was most suitable for eliminating visible contamination for both species of garlic. The first cell division was observed exceptionally after 6 days of culture

for *A. sativum* and after 5 days for *A. longicuspis* in one of three media only (Table 1) for both species, and if contamination was overcome with gentamicin. The protoplasts survived in the culture: 4 weeks for *A. sativum* and 5 weeks for *A. longicuspis* contrarily to the protoplasts cultured in the absence of antibiotic. At present we have no explanation for the absolute necessity of the antibiotic in our experiments, while few groups did not report necessity of antibiotic for surviving of cultures of A. sativum (Opatrný and Havránek, 1977; Fogher and Corti, 1982; Oosawa and Takayanagi, 1984; Havel and Novák, unpublished; Nishio et al., 1989).

3.2. Cloves protoplasts

Only Fogher and Corti (1982) used the cloves of *A. sativum* as donor material for the isolation and culture of protoplasts. The protoplasts were different in size and were quite white. The culture medium probably allowed the regeneration of a new cell wall, which however was not followed by the cell division.

3.3. Callus protoplasts

For the first time, to our knowledge, the calli derived from garlic tissues were used for initiating of protoplast cultures (Fellner and Havránek, 1993).

3.3.1. Calli production

Surface sterilized leaf basal parts of both species were placed in culture medium for callus growth induction. For further growth small calli were maintained in the dark at 25°C in the same medium as the culture of callus protoplasts (Table 1) but without mannitol, and with agar, and transferred to fresh medium every month.

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 upper limit for maintaining of explant viability. The higher concentration led to the killing of explants, to a reduction of callus induction, and moreover did not turn out to have a bigger efficiency. Despite the presence of 1.5% active chlorine, leaf explants cultured for calli induction were contaminated (Fellner an Havránek, 1993). The contamination was substantially lower for *A. longicuspis* (21%) than for *A. sativum* (78%) and was shown mainly in the course of the first five days of the culture of the explants, exceptionally later. In the majority of cases, contamination appeared as yellowish turbidity in the contact of explants with medium. Similar symptoms were observed for both species, *A. sativum* and *A. longicuspis*, during callus cultures over several months.* As expected, contamination of leaf explants was negligible (about 0.5%) and callus induction was high (Fellner, unpublished). Recently, high contamination (60%) has been reported by Rauber adn Grunewaldt (1988) for the culture of leaf explants from *A. sativum.* On the contraty, explant cultures from *A. porrum* and *A. schoenoprasum* exhibited very low contamination (9% and 0%, respectively) (Rauber and Grunewaldt, 1988),

* As a control, totally fertile genotype, *A. porrum* cv; "Elephant", was tested in the same culture conditions as the ones used for *A. sativum* and *A. longicuspis*.

results in agreement with our own observations on *A. porrum.* Thus, sterile plants (like *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 example). For these later ones, seeds should be much more free of contamination and thus the next generation will be less contaminated. This raised an interesting hypothesis as to the influence of the pathogens on the development of flower organs and more specifically on the seed production reversely.

Simultaneously, a number of papers exists concerning the induction of calli from leaves of *A. sativum,* where the problem of contamination was not mentioned (Havránek and Novák, 1973, one for all). It may be because the occurrence of contamination is associated with field conditions, season, and plant development (Lu et al., 1989). Its visible occurence in the callus culture is also partly affected by the composition of culture medium (Fellner, unpublished). In our callus cultures where visible contamination was not observed before, it was shown to be apparent later that calli contain latent inner contaminants which do not show up often and remain invisible to sight. Preliminary tests showed that they are nonphytopathogenic bacteria as in the case of the protoplast cultures of both species. It is possible that the level of contamination is low because the medium is additioned with gentamicin at the concentration of 80 mg/l. From these calli several new plants could regenerate and be transfered into soil (Fellner, unpublished).

Calli derived from leaf basal parts of *A. sativum* and *A. longicuspis,* and cultured in a medium without antibiotic were used as the source material for isolation and culture of protoplasts.

3.3.2. Behaviour of callus protoplasts

In terms of size and structure, suspensions of fresh protoplasts isolated from calli of both species were heterogenous. Viability of callus protoplasts at the start of culture was high (around 80 to 96%) compared with protoplasts isolated from leaves. Protoplasts were cultured in a liquid medium (Table 1) without antibiotic at the density $5x10^5$ protoplasts/ml. Cell wall formation was observed for both, *A. sativum* and *A. longicuspis,* over 24 hours, and better/more frequently after 35 hours of culture. A hard budding was always observed. The calli produced by leaf explants were a better source of material for protoplast isolation and culture.

Difficulty for continued growth could come from an heterogeneity of the population in terms of ploidy as it has been demonstrated by Havránek and Novák (1973) and Novák et al. (1982) that *A. sativum* calli revealed mixoploid characters. More important was the fact that we obtained more easily, more frequently and reproducibly the presence of cell division compared with culture of leaf protoplasts. For *A. sativum* the first cell division was observed after 2 to 3 days of culture but it remains a rather rare event, and moreover second cell division and colony formation were not observed. Nevertheless, callus protoplasts from *A. longicuspis* were more responsive. First cell division was observed in the course of 2 to 3 days of culture. Second cell division was observed after 7 to 12 days and

even small colonies were observed after 20 days of culture. Protoplast-derived cells of both, *A. sativum* and *A. longicuspis* survived in the culture without a next development for periods as long as 40 to 50 days.

Recent results of studies concerning the influence of antibiotic ciprofloxacin (Bayer AG, Germany) showed a significantly positive effect (in concentration range 10 to 20 mg/l) for both species in keeping with protoplast viability during the first several days of culture compared with control without antibiotic (Fellner, 1993). Ciprofloxacin is a broad-spectrum antibiotic of the quinolone series that possesses markedly greater antibacterial activity compared to other derivatives of this group. Ciprofloxacin also is known to have an inhibiting effect on mycoplasma cultures. It inhibits the bacterial DNA gyrase, an enzyme that controls the optimal configuration for the reading of the DNA. Ciprofloxacin exerts a bactericidal effect also on gram-negative organism in the resting stage (Bayer AG, Germany, Laboratory manual). In our preliminary experiments (contrarily to gentamicin), ciprofloxacin was not effective in eliminating the contamination occuring in garlic calli as it was in the elimination of contamination presented in leaf protoplast cultures (Fellner, unpublished).

Some reports cast doubt about the survival of mycoplasma or MLO for long periods of time in callus cultures derived from different species of diseased plants (Ulrychová and Petrů, 1975; Jacoli, 1978; Möllers and Sarkar, 1989). At the same time reports confirming long-term survival of these organisms in callus cultures and their transfer on regenerated plants are known (Petrů and Ulrychová, 1975, 1978; Ulrychová and Petrů, 1980). Experiments are planned to verify the possible presence of bacteria, mycoplasmas and MLO in callus and protoplast cultures of *A. sativum* and *A. longicuspis.* Future experiments should concentrate on the study of the influence of these organisms on the physiological characteristics of contaminated plants and *in vitro* cultures derived from, including protoplast cultures.

Moreover, one possible hypothesis suggests here that the blockade of cell division in the protoplast cultures could be due to the accumulation of an autotoxic sulfur compound, as alliin (or allicin and ajoene) in garlic (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 marked in the cultures of protoplasts isolated directly form leaves or differentiating cultures than for protoplasts isolated from undifferentiated callus cultures, where the content of alliin is two times lower compared with differentiating cultures (Malpathak and David, 1986). The presence of flavour precursors in undifferentiated white calli differed from the presence of flavour precursors in the intact garlic plants (Lancaster et al., 1988). These toxic products can be "neutralized" by SH-compounds like cystein, β-mercaptoethanol, glutathion or DTT (K.G. Wagner, personal communication). Simultaneously, a phenomenon was observed that in the callus cultures placed on light and regenerating shoots, the visible contamination ceases to show up (Fellner, unpublished). It could be a trend towards a hypothesis about the influence of these toxic sulfur compounds on certain suppression of latent contaminating microorganisms in *in vitro* cultures (or also in plants), generally the hypothesis about a relationship between presence of sulfur compounds and present pathogens.

4. Conclusions and perspectives

In *in vitro* cultures, plants of *Allium* genus belong to a fastidious plant group, especially for protoplast cultures. In addition, garlic *A. sativum* is not the economically most important plant available for experimentation. For this reason, research in this species is not of primary concern as compared to other economically significant plants. These facts are reflected in a small number of published papers. From this it is also evident that experiments are carried out largely empirically, demonstrating that we are still far from understanding the processes of garlic protoplast culture and regeneration. The number of papers concerning protoplast isolation procedures is higher than the number of papers studing subsequent development of protoplasts in culture (Fellner, 1991, for detailed review). Garlic protoplasts were isolated firstly from leaves of plants grown in the field or in a greenhouse. Standard procedures of regeneration and growth of garlic protoplasts are not yet available,
the protoplast culture usually ceases by the first or second cell division; sometimes small colony formation was observed (but only in very limited numbers). The reason for the cells ceasing development is still unknown.

The successful culture of protoplasts will probably be dependent upon the choice of suitable external factors such as the culture medium, physical environment and the application of new culture techniques. In spite of this, the composition of a culture medium and culture conditions do not appear to play a major role in the continued growth of the culture. Most important seemed to be the choice of the starting material, such as the plant genotype and donor tissue.

Further experiments with garlic protoplast cultures should be systematically incorporated into wider research involving the study of cell physiology, flower biology and the study of contamination by microorganisms of plants.

If attempts to control the process of the culture of protoplasts concerning plant regeneration succeed, then great possibilities would be opened for further theoretical research of the physiology of this plant as well as for possible genetic manipulation making way for understanding the problem of the pollen sterility of garlic.

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Table 1. Plant material, donor tissues and culture techniques which were used for garlic protoplasts

Plant material	Donor tissue	Culture technique	Result	Reference
A. sativum cv. "Bzenecký paličák"	green leaves and white leaves	MS[a] (LM[1] or SM[2]) +0.5 g/l casein 20 g/l sucrose 1 mg/l 2,4-D 1 mg/l kinetin 0.4M mannitol pH 5.7; light 500 lx; microdrops or Petri dishes plastic, 60 mm; 10^5-10^6 prot/ml	1st cell division	Opatrný and Havránek, 1977
A. sativum cv. "Aglio bianco piacentino"	leaf tips and cloves	B5[b] (LM or SM) +1% sucrose 0.5% glucose 9% mannitol 1 mg/l 2,4-D 0.5 mg/l NAA 0.5 mg/l BAP without inositol pH 5.8; Petri dishes, 50 mm; 10^5 prot/ml	no cell division	Fogher and Corti, 1982
A. sativum cv. "White roppen" cv. "Inhu-wase"	leaves	B5 (LM) +1% sucrose 0.5% mannitol 0-2 mg/l BAP 0-2 mg/l 2,4-D	severalfold cell division	Oosawa and Takayanagi, 1984
		LSc (LM) +1-2% sucrose 0.5 manitol 1-2 mg/l BAP 0-2 mg/l 2,4-D 0-1 mg/l NAA	no cell division	
A. sativum cv. "White roppen" cv. "Ishu-wase"	leaves	KM8pd (LM) +1% sucrose 0.5M mannitol 1 mg/l BAP 0-2 mg/l 2,4-D 0-2 mg/l NAA 0-0.5 mg/l zeatin	severalfold cell division	Oosawa and Takayanagi, 1984
		for all media: pH 5.8-6.0 28°C light 500 lx; microdrops or Petri dishes plastic, 60 mm; 105-106 prot/ml	no cell division	

(continued)

Plant material	Donor tissue	Culture technique	Result	Reference
A. sativum cv. "Bzenecký paličák"	white leaves	BDS[e] (LM) + 58 mM sucrose 0.2M mannitol 0.2M sorbitol 1 g/l casamin acid 10 µM kinetin 10 µM IAA 5 µM 2.4-D	no cell division	Novák et al., unpublished
A. sativum cv. ?	leaves	MS or KM8p (?) + 0.3-0.5M mannitol, glucose or surcrose auxins cytokinins (concentration?)	no cell division	Nishio et al., 1989
A. sativum cv. "Moravan" (virus-free)	basal parts of leaves	MS or BDS (LM) + 2% sucrose 9.1 µM 2,4-D 2.2 µM BAP mannitol to 560-600 mOs/kg	no cell division	Fellner and Havránek, 1993 (submitted)
A. sativum cuspis (wildtype, virus free)		BDS (LM) + 2% sucrose 1 mM 2,4-D 5 mM kinetin mannitol to 560-600 mOs/kg	1st cell division (for both species)	
		for all media: pH 5.7 with or without gentamicin, 25°C; dark; Petri dishes plastic, 60 mm		
	calli	BDS (LM) + 2% sucrose 1mM 2,4-D 5 mM kinetin mannitol to 580-660 mOs/kg; 25°C; dark; Petri dishes plastic, 60 mm	1st cell division (for *A. sativum*) small cell colonies (for *A. longicuspis*)	

[a]Murashige and Skoog medium (Murashige and Skoog, 1962)
[b]Gamborg medium (Gamborg et al., 1968)
[c]Linsmaier and Skoog medium (Linsmaier and Skoog, 1965)
[d]Kao and Michayluk medium (Kao and Michayluk, 1975)
[e]Dunstan and Short medium (Dunstan and Short, 1977)
[1]Liquid medium
[2]Solid medium

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