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Influence of the Antibiotic Ciprofloxacin on Culture of *Allium longicuspis* Callus-derived Protoplasts

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A major problem of *in vitro* plant culture techniques is chronic contamination by microorganisms. Calli derived from basal parts of leaves of *Allium longicuspis* Regel (Alliaceae) and cultured in a medium without antibiotic contain most probably latent contaminating microorganisms. These calli were used as the source material for isolation and culture of protoplasts. Isolated protoplasts were cultured in the presence of the antibiotic ciprofloxacin, and the protoplast viability, cell wall regeneration and cell division were studied as a function of the antibiotic concentration. Whatever the antibiotic concentration, protoplast-derived cells kept significantly higher viability for at least 3 weeks compared with those cultured without antibiotic. As to cell wall regeneration after 2 d, it was not affected by the antibiotic except at the highest concentration tested (100 mg l^{-1}). Sporadic first cell division was observed after 2–6 d of culture in the presence of ciprofloxacin while, in its absence, cell division was never apparent before 10 d of culture.

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Key words: *Allium*, bacteria, cell division, cell wall regeneration, ciprofloxacin, contamination, garlic, mycoplasma, protoplast culture, viability.

INTRODUCTION

Wild species of garlic *Allium longicuspis* Regel, a proposed progenitor of garlic *Allium sativum* L., is a sterile plant which does not form seeds and thus reproduces exclusively by vegetative propagation like garlic *A. sativum* L.

A microorganism phenomenon (mycoplasma or MLO phenomenon) has been postulated to cause a disease of the tapetum in garlic (Konvička, 1972, 1973, 1978, 1984). In this connection, contamination of garlic explants *in vitro* was recently described (Rauber and Grunewaldt, 1988; Fellner and Havránek, 1994), as well as the chronic contamination by viruses of garlic plants (Bos, 1983, and references therein). These points raise the questions whether such infections lead *in planta* to garlic sterility and *in vitro* to the recalcitrant physiological characteristics of cultures. The absence of sexual reproduction impoverishes garlic's genetic variability. For this reason, garlic regeneration from protoplasts is a current goal and this step is a prerequisite to overcoming the problems involved in sexual reproduction of *Allium* plants using somatic, gametosomatic hybridization or direct transformation of protoplasts.

Reports concerning the culture of protoplasts from garlic plants are few (Fellner, 1993, for detailed review) and moreover the protoplast cultures of garlic have not been very successful. Consequently, only two examples of *Allium* plant regeneration are available, from mesophyll protoplasts of onion (*A. cepa*) (Wang, Xia and Wang, 1986) and from cell suspension protoplasts of leek (*A. porrum*) (Schum, Junge and Mattiesch, 1994); both are related to garlic but are pollen fertile plants. It appears that garlic cultures *in vitro* have a chronic contamination by unknown microorganisms, probably of a latent nature (Fellner and Havránek, 1994).

The aim of this work was to describe behaviour of protoplasts in the presence of the broad-spectrum antibiotic ciprofloxacin which possesses antibacterial and anti-mycoplasma activity.

MATERIALS AND METHODS

Plant material and callus cultures

Wild type *Allium longicuspis* Regel, the proposed garlic progenitor (Vvedensky, 1944; Jones and Mann, 1963, both cited in Brewster and Rabinowitch, 1990) (collected in area of Issyk-Kul lake in Uzbekistan), from the germplasm collection of the former Research Institute of Vegetable Growing and Breeding in Olomouc, Czech Republic, was used for the experiments. Like *A. sativum*, *A. longicuspis* is a sterile plant but, unlike *A. sativum*, it has fertile pollen.

Cloves of virus free plants, originally obtained from meristem-tip cultures (Havránek, 1972), were planted into pots with artificial substrate ('Perlit'). They germinated and plants grew in the greenhouse under natural light conditions at the temperature of 23 to 27 °C. Calli from basal parts of leaves derived and used in 1991 (Fellner and Havránek, 1994) were maintained in this work under the same conditions as in the previous experiments, namely, in the dark at 25 °C on BDS medium (Dunstan and Short, 1977) supplemented with 30 g l^{-1} sucrose, 1·0 μM 2,4-D and 5·0 μM kinetin (pH 5·7, adjusted by 1 N NaOH) and solidified by

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agar (0·8%); these were used as the source of protoplasts. They were transferred to fresh medium every month.

Isolation and culture of protoplasts

The calli, 1 week after a transfer to fresh medium and free of visible contamination, were cut off in small pieces and placed into 100 mm Petri dishes containing the enzyme solution (10 ml for 5 g f.wt). The 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) $CaCl_2.2H_2O$ (pH 5·6) (Fellner and Havránek, 1994). The osmolality was adjusted to 600–690 mOs kg^{-1} H_2O with mannitol. The enzyme solution was sterilized by filtration through a 0·2 μm filter. Digestion was done in the dark at 25 °C for 14 h, without shaking.

The protoplast suspension was strained through a 42 μm mesh nylon filter and centrifuged at 70 *g* for 5 min. The pellet was resuspended in a washing medium (WM) (pH 5·6) containing 0·4 M glucose and 18 mM $CaCl_2.2H_2O$, and further purification by gradient was performed. Protoplasts (2 ml) were partitioned by 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 debris pelleted and purified protoplasts were layered at the glucose/sucrose interface. They were collected and washed once more in WM by a centrifugation at 50 *g* for 6 min. The final pellet was resuspended in a culture medium at a density of 5×10^5 protoplasts per millilitre.

Protoplasts were cultured in 60 mm plastic Petri dishes in the dark at 25 °C in liquid BDS medium supplemented with 1·0 μM 2,4-D and 5·0 μM kinetin (Fellner and Havránek, 1994). Osmolality of medium was adjusted with mannitol to 580–660 mOs kg^{-1} H_2O and the pH was adjusted by 1 N NaOH to 5·7 before autoclaving. In addition, protoplasts were cultured in the presence of the antibiotic ciprofloxacin (Ciprobay®, Bayer, Germany) in concentrations from 5 to 100 mg l^{-1} at the same pH, and the protoplast viability, cell wall regeneration and cell division were studied as a function of the antibiotic concentration. Viability of protoplasts was determined by fluorescein diacetate (FDA) staining (Widholm, 1972) and cell wall regeneration by the UV-excited optical brightener Rylux BSU (Synthesia, Pardubice-Semtín, Czech Republic) staining (Hejtmánek, Doležel and Holubová, 1990) (Rylux BSU was kindly provided by Professor M. Hejtmánek). Both cell viability and cell wall regeneration were observed by fluorescent inverted microscopy (Leitz, Austria).

RESULTS

The calli of *A. longicuspis* derived in 1991 were used as the source material for isolation of protoplasts as in previous experiments in 1991 (Fellner and Havránek, 1994). Some of those calli (about 25%) showed visible contamination throughout culture. In the remaining calli where visible contamination was not first observed, it was later shown that calli contain latent inner contaminants which often do not show up and remain cryptic. The origin of contaminating microorganisms has not yet been determined. However, this visible infection can be suppressed by the culture of calli on medium enriched with gentamicin (Fellner, unpubl. res.). Calli which did not show visible contamination when cultured without any antibiotic were used as the source material for the experiments presented in this paper.

TABLE 1. *Regeneration of new cell wall of* A. longicuspis *protoplasts (%) cultured in the presence of ciprofloxacin in culture medium. Data indicate cell-wall-regenerating protoplasts as percent of total protoplasts (means ± s.e., P = 0·05). Cell wall presence at time 0 (fresh isolated protoplasts) was 1 ± 2·1%*

Ciprofloxacin (mg l^{-1})	Time of culture (d)	
	1	2
0	36·8 ± 6·5	51·7 ± 14·8
5	38·9 ± 11·1	52·9 ± 5·9
10	32·2 ± 5·2	51·0 ± 5·9
20	35·7 ± 10·7	51·4 ± 10·5
100	17·0 ± 7·9*	28·8 ± 11·5

* Significant difference to control (absence of ciprofloxacin in culture medium).

The yield of protoplasts isolated from the calli after 14 h of digestion varied from $5·8 \times 10^6$ to 7×10^6 protoplasts g^{-1} f.wt depending on the experiment, and the average viability of fresh protoplasts was 69·1% (s.e. ± 7·5%). Protoplasts cultured without as well as with ciprofloxacin synthesized new cell wall early, and after 24 h more than 35% of protoplasts showed (by fluorescence) presence of new cell wall (Table 1). After 2 d of culture, most viable protoplasts had cell wall, with the exception of protoplasts cultured in the presence of 100 mg l^{-1} ciprofloxacin where the cell wall formation was partly but significantly inhibited. More distinctly, the viability of protoplast-derived cells was increased by the presence of antibiotic (Table 2). Differences among various concentrations of antibiotic were slight and not statistically significant. However, as shown in Table 2, the viability of cells after 21 d of culture in the presence of the antibiotic was significantly higher than the control for all tested concentrations. From the values in Table 2 one can calculate the decline of the viability (Fig. 1) (when we equate the viability of fresh protoplasts with 100%) during several periods of the protoplast culture. The first period is from days 0 to 3, the second period from days 3 to 13 and the last one from days 13 to 21. During the first period, the loss of viability per day was highest for all tested conditions. The viability of fresh protoplasts cultured in the absence of the antibiotic decreased during those first 3 d by about 42·3%, during the next 10 d by about 39·8% and then, during the last 8 d by about 15·5%: altogether, total viability declined during 3 weeks by about 97·6% (in Table 2, the level of viability fell from 69·1% of fresh protoplasts to 1·7% after 21 d). Compared with the control, the loss of viability was less during the first 3 d at all concentrations of ciprofloxacin used, with the best at 10 mg l^{-1} ciprofloxacin (24·2%). Loss of viability in protoplast-derived cells cultured during 3 weeks in the presence of the antibiotic was always lower compared with the control, fluctuating between 84·8 and 87·9% (5 mg l^{-1} to 100 mg l^{-1} ciprofloxacin). No toxic

TABLE 2. *Viability of the protoplast-derived cells of* A. longicuspis *in the presence of ciprofloxacin during 21 d of culture. Data indicate viable protoplasts or protoplast-derived cells as percentage of total protoplasts (means ± s.e.,* P = 0·05)

Time of culture (d)	Ciprofloxacin concentration (mg l^{-1})				
	0	5	10	20	100
0	69·1 ± 7·5[a]	69·1 ± 7·5[a]	69·1 ± 7·5[a]	69·1 ± 7·5[a]	69·1 ± 7·5[a]
1	51·6 ± 5·0	56·4 ± 6·9	60·2 ± 6·1	54·4 ± 9·0	53·1 ± 6·7
3	39·9 ± 9·6	47·4 ± 13·6	52·4 ± 11·3	49·7 ± 17·1	42·7 ± 10·5
7	25·9 ± 4·0	31·6 ± 6·9	35·8 ± 5·6*	32·4 ± 6·9	32·5 ± 10·9
13	12·4 ± 5·4	23·2 ± 9·0	24·5 ± 7·3	21·7 ± 8·6	17·5 ± 12·8
21	1·7 ± 0·6	10·5 ± 2·1*	10·2 ± 2·3*	10·1 ± 2·9*	8·4 ± 12·8*

[a] Viability of fresh isolated protoplasts.
* Significant difference to control (absence of ciprofloxacin in culture medium).

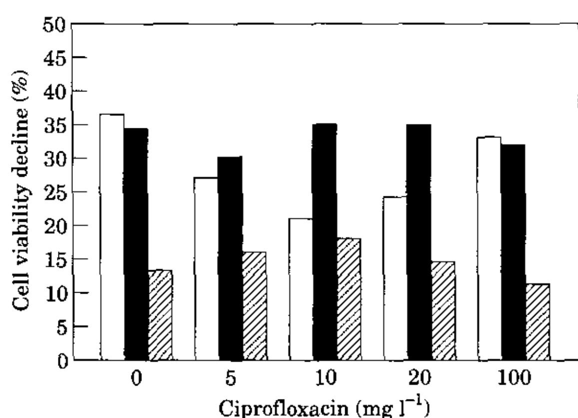

FIG. 1. Loss of viability of *A. longicuspis* protoplast-derived cells during periods of their culture as a function of ciprofloxacin concentration. Values are in percent related to the viability of fresh protoplasts equated with 100% (□) first period, 0–3 d; (■) second period, 3–13 d; (▨) third period, 13–21 d.

effect of ciprofoxacin on cell viability was evident in our experimental conditions.

Ciprofloxacin did not show any effect on percentage of divided cells. Protoplasts divided exceptionally, at a very low rate not exceeding 0·01%. In all experiments which have been done, the protoplasts in the absence of antibiotic divided not before day 10 of culture. However, in the presence of ciprofloxacin at 5 and 10 mg l^{-1}, the protoplasts divided from the second day of culture. With 20 or 100 mg l^{-1} ciprofloxacin, protoplasts divided largely between days 3 and 6 of culture and the first division in presence of the antibiotic was always before day 6 of culture. Further cell division was never observed in any of the tested conditions.

In all experiments, visible contamination of the protoplasts was not apparent, neither in the absence nor in the presence of ciprofloxacin. In contrast, the source calli were sooner or later always found to be visibly contaminated.

DISCUSSION

Plants of the *Allium* genus are fastidious for culture *in vitro*. Protoplast cultures of garlic have not yet been really successful (see Fellner, 1993 for review) and have mainly involved protoplasts isolated from leaves and mesophyll. In our previous experiments (Fellner and Havránek, 1994), leaf protoplasts showed chronic contamination, they did not divide and were soon dead. However, protoplasts cultured in the presence of the antibiotic gentamicin did divide, albeit exceptionally. Calli were then used as source material in the hope of minimizing cryptic contamination. Unlike leaf protoplasts, callus-derived protoplast cultures never showed visible contamination. They divided (at a low rate), and occasionally formed small cell colonies (Fellner and Havránek, 1994). However, the calli used for these experiments sooner or later revealed contamination. The nature of contamination, whether chronic or cryptic, is uncertain, but preliminary tests showed the presence of some gram-positive and gram-negative bacteria (Kůdela *et al.*, unpubl. res.), and/or bacterium of *Bacillus* genera (Vála, unpubl. res.). In other more thorough tests, the colonies of bacteria *Bacillus circulans* and *Staphylococcus xylosus* (and *S. warneri*) were identified from visibly-contaminated calli of *A. longicuspis* and *A. sativum*, respectively (Kneifel *et al.*, unpubl. res.). On the other hand, the bacterium *Serratia marcescens* and some fungi were found in calli of both *A. longicuspis* as well as *A. sativum* (Smith, unpubl. res.). There appear to be latent inner contaminants which did not unduly modify physiological characteristics of calli (cultures *in vitro* generally) and of intact plants. This presumption is supported by several reports showing the presence of contaminating microorganisms in intact garlic plants (Konvička *et al.*, 1978; Bos, 1983) and in cultures *in vitro* (Rauber and Grunewaldt, 1988).

Accordingly, the present work with antibiotic ciprofloxacin was undertaken to study its effect on some characteristics of garlic protoplasts, such as the cell wall regeneration, cell viability and cell division. Ciprofloxacin is a broad-spectrum antibiotic of the quinolone series that possesses broad antibacterial activity. It is also known to have an inhibitory effect on mycoplasma. Ciprofloxacin inhibits the bacterial DNA gyrase which controls the optimal configuration for reading of DNA. Disturbance of this process causes the bacterial metabolism to collapse as the essential information on the chromosome cannot be transcribed. Consequently, pathogenic microorganisms that are in the proliferation phase are killed off rapidly. In addition, ciprofloxacin exerts a bactericidal effect also on

gram-negative organisms in the resting stage (Laboratory Manual, Bayer AG, 1990).

In our earlier unpublished experiments, ciprofloxacin was not effective in eliminating the contamination occurring in garlic calli but it at least had no toxic effect on callus growth. Further, ciprofloxacin was effective in the elimination of contamination presented in the leaf protoplast cultures and it did not show any toxic effect on protoplast viability. In the same way, ciprofloxacin in this work had no toxic effect on viability of callus-derived protoplasts and indeed, in the presence of ciprofloxacin in the viability of cells was significantly higher (approximately four-fold) for each concentration compared with the control after 21 d of culture. The decline of cell viability in the first period (0–3 d) at 10 mg l^{-1} ciprofloxacin was about half the control (Fig. 1). Some inhibitory effect on regeneration of new cell wall was observed at 100 mg l^{-1} ciprofloxacin. No effect on the level of cell division could be seen, but the timing of the first division was shortened: in the absence of ciprofloxacin, the protoplast-derived cells divided after 10 d, in its presence they divided not later than day 6 of culture. Although in the previous experiments (Fellner and Havránek, 1994) the viability of fresh protoplasts isolated after 19 h digestion was similar to that after 14 h here, the cell viability decline in previous experiments was about 24% in the first period instead of 42% in this work. As to cell division, in previous experiments the protoplasts had divided in the course of 2–3 d of culture in the absence of the antibiotic and second cell division and cell colony formation were observed, while in this work the protoplasts divided after 10 d and only a first cell division was observed. It is now evident that protoplasts derived from long term garlic calli used in this work had partly lost their capacity for viability and cell division compared with protoplasts prepared from freshly derived calli such as used in the previous experiments, even though the capacity of these long term calli to regenerate plants is still high because they readily form shoots and roots in culture medium in the light. A decline of regeneration ability in long term cultures is a common phemomenon connected probably with their heterogeneity in terms of ploidy, as has been demonstrated for garlic calli (Havránek and Novák, 1973; Novák, Havel and Doležel, 1982), and in rose where development of aneuploid and polyploid subpopulations is correlated with the loss of this regeneration ability (Moyne *et al.*, 1993).

There are no reports of any effect of ciprofloxacin or any other antibiotic on garlic cultures *in vitro*. However, several reports show a stimulatory effect of antibiotics on plant tissue cultures seen, for example, in stimulation of metabolic pathways, growth ratio, embryogenesis as well as in regeneration (Santos and Salema, 1985; Nakano and Mii, 1993). Although the relevant mechanism is uncertain, several possible explanations exist. First, antibiotics may act as a stress and thus induce somatic embryogenesis (Nakano and Mii, 1993, and references therein). The second possible explanation is that these compounds could mimic plant growth regulators. Santos and Salema (1985) reported that penicillins enhanced growth of callus cultures of *Sedum telephium*. Similarly, penicillins potentiated growth of protoplast-derived colonies of *Nicotiana plumbaginifolia* (Pollock, Barfield and Shields, 1983). Penicillins stimulated callus growth and organogenesis in *Antirrhinum majus* (Holford and Newbury, 1992) and it was shown that these antibiotics broke down in culture medium to give physiologically active levels of the auxin phenylacetic acid. When a compound (also antibiotic) is not toxic at higher concentrations and, in spite of this, has stimulatory effect on cultures, then it is possible that the compound is being metabolized with some nutritive value. In our experiments, on the one hand, the contamination was not yet evident in the callus culture used as source. Nor was contamination evident in the protoplast cultures under the same conditions as calli, using liquid medium containing mannitol, instead of solid medium free of mannitol (used for calli). The contamination in callus cultures was evident only after several subcultures, so maybe the 3 weeks of protoplast culture was insufficient for assessment of contamination. Yet on the other hand, if we tried to isolate protoplasts from visibly contaminated callus cultures, then protoplast cultures also showed visible presence of microorganisms. At least, enzymatic digestion did not kill microorganisms contaminating callus cultures (or did not kill them all). Moreover, protoplast cultures derived from visibly non-contaminated calli could also contain microorganisms not yet evident because of their low concentration. Ciprofloxacin, even when applied on visibly non-contaminated calli, did not suppress the contamination in callus cultures which eventually showed several days or weeks later. Therefore, if ciprofloxacin is not strictly biocidal, it could directly influence protoplast cultures nutritively in some way. Yet the effect of ciprofloxacin was essentially not concentration-dependent (the highest concentration of ciprofloxacin was inhibitory only in the case of cell wall regeneration). Therefore, we propose that ciprofloxacin acts by lowering the concentration of viable microorganisms in the callus and protoplast cultures, and notably their localization in the tissues *in vitro* such that contamination is held cryptic or sub-clinical.

It is known that activity of antibiotics is affected by pH. All our experiments have been done at pH 5·6–5·7, whereas ciprofloxacin's general optimal activity is pH about 7·0–8·0, values which would be lethal to plant cells (Pollock *et al.*, 1983). This may explain the non-toxicity of ciprofloxacin to microorganisms at relatively high concentrations (20, 100 mg l^{-1}) used in these experiments. It is urgent to identify in detail the microorganisms in *A. longicuspis* callus cultures and to test the effective conditions for their control.

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