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ARTICLE

Protoplast isolation from *Solanum lycopersicum* L. leaf tissues and their response to short-term NaCl treatment

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ABSTRACT Protoplasts were isolated from young leaves of tomato (Solanum lycopersicon L. cvar Rio Fuego). The optimum conditions for protoplast isolation was established by using 2% cellulose R-10 and 0.5% macerozyme R-10 dissolved in 0.4 M sucrose-K3 solution for 12 h cell wall digestion. In order to induce salt stress, the mannitol content of the buffer was partially replaced by NaCl to get an isoosmotic incubation solution containing 100 mM NaCl. It can be concluded that the number of protoplast in unit volume counted by Bürker chamber did not decrease significantly compared to controls due to salt treatment upto 5 hours, but the viability of cells decreased by 55% using fluorescein diacetate staining. Hundred mM NaCl simultaneously enhanced the generation of reactive oxygen species in tomato leaf protoplast. This means that decreases in fluorescein fluorescence is a good and sensitive parameter for the measurement of Na^+-induced decrease in cell viability and cell death in protoplast suspensions.
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KEY WORDS

Solanum lycopersicum
protoplast suspension
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Salinity is one of the most important environmental stresses. Agricultural productivity is severely affected by soil salinity, therefore it is very important to study the effects of salt stress on cell level. Salt stress disrupts the homeostasis of intracellular ion concentrations. These changes lead to the production of the reactive oxygen species (ROS) and salt stress finally may cause cell death. High salinity also causes changes in water status of cells and it leads to growth arrest (Zhu 2001).

Protoplasts are especially good models to study physiological processes in plant cells. They can be used for in vitro manipulation of tomato to improve salt tolerance (Dorion et al. 1999) or for the analysis of Na^+ sequestration in the cell compartments of protoplasts prepared from salt tolerant and salt sensitive rice cultivars (Kader and Lindberg 2005). There are only few reports on the direct treatment of protoplasts with supraoptimal concentrations of Na^+ (Aditya and Baker 2003), because it has several problems. Number and viability of protoplast may decrease with increasing age of the preparation and with the concentration of Na^+. Thus, a reliable counting of viable cells after treatments is very important.

Fluorescein diacetate (FDA) is a molecule which is widely used to determine the viability of tomato suspension cells (De Jong et al. 2000; Yakimova et al. 2007), tobacco protoplasts (Saunders et al. 1986) and orchid seeds (Pritchard 1985). The principle of staining with FDA is that the non-polar molecule can get through the membrane, so the cells take it up. The non-specific esterases, which are located in the intracellular space, can than hydrolyse the ester bonds, thus non fluorescent FDA becomes fluorescent free fluorescein. Fluorescein is a polar molecule which remains in the cytoplasm, because it can not pass through the intact plasma membrane (Rotman and Papermaster 1966). In contrast with the living cells the dead cells can not hydrolyse the FDA molecule because of the inactivity of the enzymes or even if the cells hydrolyse the dye, the fluorescein simply effuses from the cells. Thus in this staining procedure the viable cells show fluorescence, and the dead cell do not.

In our experiments the counting of cell number in Bürker chamber and the staining with FDA was used to see, how the viability of the tomato leaf protoplasts is affected by 100 mM NaCl as a function of time. We were also interested in the generation of ROS especially of H_2O_2 by NaCl in the protoplast in connection with their viability. Our aim was also to show that decrease in the fluorescence after FDA staining of constant number of tomato protoplast is a suitable parameter of Na^+-induced loss of viability or cell death.

Materials and Methods

The leaves of 3-week-old tomato plants (Solanum lycopersicum Mill. L. cvar. Rio Fuego) were used for protoplast preparation. The seeds were germinated on 26°C, for three days in the dark. The seedlings were grown in a greenhouse in perlite and after seven days they were cultivated in hydroponic culture for two weeks. The nutrient solution contained 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.5 mM

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Na_2HPO_4, 0.5 mM KCl, micronutrients (10^{-6} M $MnSO_4$, $5 \cdot 10^{-7}$ M $ZnSO_4$, 10^{-7} M $MCuSO_4$, 10^{-7} M $(NH_4)_6Mo_7O_{24}$, 10^{-5} M H_3BO_4) and $2 \cdot 10^{-5}$ M Fe-EDTA at pH=5.8. The plants were grown for 12 hours in the light and 12 hours in the dark. The light intensity and relative humidity were 300 µmol m^{-2} s^{-1} and 55-60%, respectively.

Protoplast preparation from tomato leaves

Protoplasts were isolated from the young, terminal leaves of tomato plants. The leaves were cut with scissor and were put upside down into Petri dishes containing 0.4 M sucrose-K3 solution (Nagy and Maliga 1976). Before sucrose was added to the K3 solution (sucrose-K3) it was adjusted to pH 5.5. The leaves were cut into 2-3 mm wide segments and the midrib was cut out. Leaves prepared in this way were put standing straight up into another Petri dish containing 0.4 M sucrose-K3 solution. They were incubated for ten minutes in the solution at room temperature. The solution was then replaced with 15 ml of the enzyme solution, which contained 2% cellulose R-10 (Sigma-Aldrich, St. Louis, MO) and 0.5% macerozyme R-10 (Sigma-Aldrich, St. Louis, MO) dissolved in 0.4 M sucrose-K3. After an overnight incubation at 27°C the enzyme solution was filtered with Pasteur pipette into Erlenmeyer flasks. Then the content of the flasks was placed into Wasserman tubes and a layer of 2-3 ml of W5 washing solution was stratified on the protoplast solution (1L of W5 solution contained: 9 g NaCl, 18.4 g $CaCl_2 \cdot 2H_2O$, 0.8 g KCl, 1 g glucose). Before use it was adjusted to pH=5.8. The Wasserman tubes were centrifuged at low speed for five minutes. The protoplasts were clustered between the two fluid layers creating a ring, and were transferred into new Wasserman tubes. In these new tubes W5 buffers were stratified again and protoplasts were centrifuged at the lowest speed for another five minutes. The protoplasts settled on the base of the tube and the washing solution was replaced with a buffer containing 525.6 mM mannitol, 12.5 mM Na-acetate, 5 mM $CaCl_2$, pH=5.8. The protoplasts were treated with 100 mM NaCl also, in this case we replaced the washing solution with a buffer containing 400 mM mannitol, 12.5 mM Na-acetate, 5 mM $CaCl_2$ and 100 mM NaCl, pH=5,8.

Determination of cell number using Bürker chamber

The protoplasts isolated from young tomato leaves were counted with the Bürker chamber and were analyzed for viability. The viable tomato protoplasts have intact plasma membrane so the cells are spherical, on the contrary, the dead cells have abnormal form, because of the loss of membrane integrity. The cells were counted with a microscope with enlargement of 120. We counted the cells in Bürker chamber, and the percentage of viable cells and the number of cells in 1 ml suspension were determined.

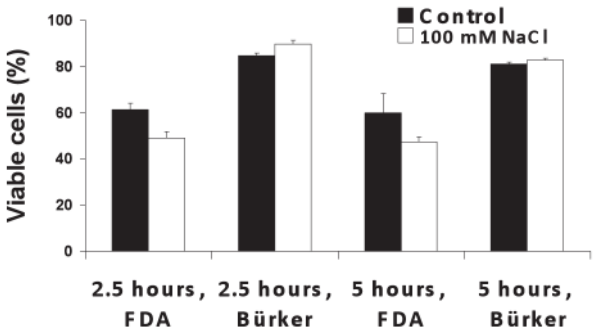

Figure 1. The effect of 100 mM NaCl on the percent of viable protoplasts counted with Bürker chamber or with FDA staining. Means±SD, n=10 for Bürker chamber detection, and 300 cells for FDA staining.

Determination of protoplast viability

Ten µM FDA solution (Sigma-Aldrich, St. Louis, MO) was used for the staining of protoplasts. After 5 minutes of incubation in the FDA dye, the protoplasts were washed for 5 minutes in the W5 buffer and the pictures about the fluorescence of protoplasts was measured with a fluorescence microscope, type Zeiss Axiovert 200M. The excitation wavelenght was λ=495 nm, and the measuring was done at 515 nm (Räthel et al. 2003.). Three randomly placed pictures where taken from the samples with a digital camera (Axiocam HR) and 773 ms exposition time and the pixel intensity was determined on 300 cells. The viability of the protoplasts was analyzed with the Axiovision 4.5 program.

Determination of reactive oxygen species

For the detection of ROS, the protoplasts were dyed with 0.01 µM 2,7-dichlorofluorescein diacetate (H_2DC-FDA) (Sigma-Aldrich, St. Louis, MO) dissolved in MES/KCl buffer for 20 minutes at 37°C, in darkness (Allan and Fluhr 1997). Different derivatives of the dichloro-dihydrofluorescein dye have been used for the detection of ROS *in vivo* (Dickens et al. 1992). These have been reported to react preferentially with $O_2^{\cdot-}$ and peroxynitrite ($ONOO^-$), but they are otherwise

Table 1. The number of viable cells counted in Bürker chamber 2.5 or 5 hours after the isolation of protoplasts in control samples and after 100 mM NaCl treatment .The cell number were calculated for 1 ml protoplast suspension (Means±SE, n=10). There were no significant differences between control and salt-treated samples.

Treatment	2.5 hours (living cells %)	5 hours (living cells %)
Control	625000 ± 180769 (92%)	575000 ± 153292.7 (86.91%)
100 mM NaCl	612500 ± 141888 (88.95%)	575000 ± 117175.5 (91.31%)

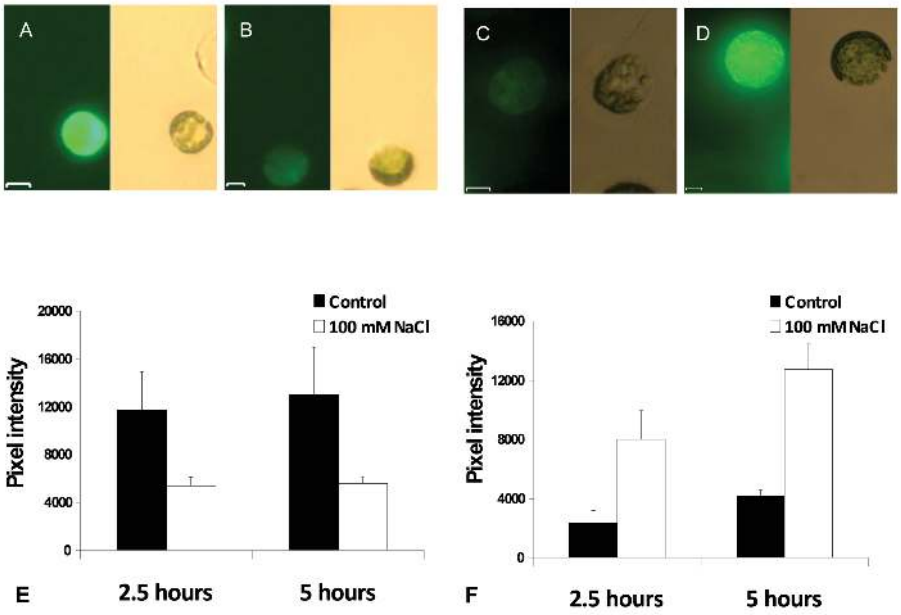

Figure 2. The green fluorescence of tomato protoplasts in control samples (A,C) and in the presence of 100 mM NaCl (B,D) after FDA staining for viability (A,B), and H_2DC-FDA staining for H_2O_2 (C, D) 5 h after protoplast isolation. Mean values±SD of the pixel intensities of protoplasts after FDA (E) and H_2DC-FDA (F) staining in control and 100 mM NaCl-treated samples. The FDA fluorescence indicate the viability of cells and that of H_2DC-FDA shows ROS production. Bars = 5 µm.

relatively unspecific, and their reaction with H_2O_2 cannot be excluded.

Results and Discussion

Protoplasts are considered very important experimental material for genetic transformation, somatic hybridisation, in vitro propagation of horticultural crops, the experimental methods often used for improving salt tolerance of crop plants, but they are rarely used for the investigation of the short-term physiological effects of abiotic stressors, such as high salinity. The protoplast yield depends on various factors such as the plant age, concentration of hydrolytic enzymes and incubation time.

To optimize the production of protoplasts, different factors, such as composition of enzyme solution, incubation period and the age of plants were studied. Good yield of protoplasts were obtained from the youngest leaf of three-week-old plants, with an enzyme solution of 2% cellulose R-10 and 0.5% macerozyme R-10 dissolved in 0.4 M sucrose-K3 solution and 12 h of cell wall digestion.

To find out if tomato protoplasts are suitable for the investigation of salt stress caused by 100 mM NaCl, we replaced the mannitol in the incubation buffer solution to NaCl. After the preparation of protoplasts, the cell number was determined as a function of time with Bürker chamber in order to determine the stability of the protoplast solution in control samples and during stress conditions. Initially the protoplast solution contained about 600000 cells in 1 ml volume. It was found that more than 80% of protoplasts were living five hours after the isolation (Table 1.).

The number of intact, spherical protoplasts were not significantly different between control and NaCl-treated samples 2.5 and 5 hours after protoplast isolation based on counting in a Bürker chamber.

The cell viability after staining with FDA was also measured as a percentage of living cells in the total number of cells. The total cell number and the number of fluorescent cells were determined on the same microscopic area. Comparing 300 cells, 55-60% of the protoplasts were viable five hours after protoplast isolation (Fig. 1). The decreases in the fluorescence of stained cells expressed in pixel intensity showed that the protoplasts treated with the 100 mM NaCl exhibited a significant decrease in cell viability (Fig. 2).

The mean pixel intensity of protoplasts decreased, when they were treated with 100 mM NaCl. This means, that before the disruption of protoplasts, salt stress decreases the viability of the cells. Protoplasts exhibited increased fluorescence under 100 mM NaCl treatment after staining with H_2DC-FDA. Increase in ROS production is a normal reaction of the cells under salt stress. Due to the increased level of H_2O_2, protoplasts considerably lose their viability but maintained the integrity of membranes and spherical shape for 5 hours. We can conclude that the loss of vitality of protoplast under NaCl stress can be demonstrated well with the decrease or loss of

the fluorescence after FDA staining, and tomato protoplasts are good models to demonstrate the effect of salt stress.

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