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In Vitro Selection and Strawberry Plant Regeneration for Developing Resistance to *Botrytis cinerea* Pers., *Phytophthora cactorum* Leb. *et* Cohn (Schroet) and Salinity Stress

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ABSTRACT

In vitro screening techniques were employed to obtain strawberry (*Fragaria* × *ananassa* Duch.) lines with resistance/tolerance to environmental stressors. The aim was to select genotypes resistant to *Phytophthora cactorum* and *Botrytis cinerea* and tolerant to chloride salinity. Callus tissues were screened for tolerance on MS medium, supplemented with 2 mg l^{-1} indole-3-butyric acid (IBA), which contained selective agents: either monthly culture filtrate of one of the two pathogens at 10, 20 and 30% or 0.05, 0.10 and 0.15 M sodium chloride. Tolerant tissues were used for adventitious shoot regeneration on MS medium with 2 mg l^{-1} 6-benzylaminopurine (BAP), 0.5 mg l^{-1} IBA and 0.2 mg l^{-1} gibberellic acid (GA_3), which were then micropropagated on MS medium supplemented with 1 mg l^{-1} BAP and 0.1 mg l^{-1} IBA. Rooting was achieved on MS medium supplemented with 0.5–1 mg l^{-1} IBA. The obtained plants were acclimatized to *in vivo* conditions in a plastic greenhouse and subsequently planted in the field. Tests for disease resistance were conducted using artificial leaf inoculation with spore-mycelium suspensions of the phytopathogens. Three methods were employed to assess tolerance to salinity: (1) evaluation of growth parameters on selective media containing 0.05 and 0.1 M NaCl, and determination of propagation coefficient and leaf necrosis scores, (2) laser analysis of tissue microstructure (LAM) and (3) leaf diagnostics. Lines exceeding the level of resistance to *P. cactorum* and *B. cinerea* ('Lvovskaya Rannyaya', 'Urozhainaya CGL' and 'Zolushka'), and those with superior tolerance to salt toxicity ('Feierverk', 'Lvovskaya Rannyaya', 'Urozhainaya CGL' and 'Zolushka') were selected. In addition to either trait, the most promising lines produced high yield and large fruit.

Keywords: strawberry (*Fragaria* × *ananassa*), laser analysis, leaf diagnostics, phytopathogenic fungi, salt stress, selective media, tissue selection

Abbreviations: 2,4-D, 2,4-dichlorophenoxy-acetic acid; **BAP**, 6-benzylaminopurine; **GA_3**, gibberellic acid; **IBA**, indole-3-butyric acid; **IFS**, index of functional state; **LAM**, laser analysis of tissue microstructure; **PAM**, pulse amplitude modulation; **PCD**, programmed cell death; **PPFD**, photosynthetic photon flux density; **ROS**, reactive oxygen species

INTRODUCTION

In addition to high yield and fruit quality, modern strawberry cultivars must be tolerant to unfavourable environmental factors. Biotechnological approaches, using cell and tissue selection, are commonly employed to accelerate the recovery of lines with such valuable traits. These methods employ selective media as a selection pressure tool to select cells and calli with valuable somaclonal changes for plant regeneration. As these methods require a moderate working space and allow screening of large amount of plant material under strictly controlled conditions, plants with desirable traits can be obtained faster at lower labour costs, besides eliminating need of field experiments.

Extensive variability of characters in callus cells is a peculiarity encouraging tissue selection. The wide-ranging variability, usually called somaclonal, is influenced by conditions of cultivation during cell dedifferentiation. Pre-existing genetic variation in somatic cells (Walbot 1985), single gene mutations aneuploidy and transposable elements, cytogenetic changes, DNA methylation and plant hormones pressure in media contribute to the somaclonal variability (Evans *et al*. 1984; Evans 1987; Dolgykh and Shamina 1991; Konstantinov and Rivkin 1991; Jain and Pehu 1992; Jain 1997a, 1997b). Unlike epigenetic changes, somaclonal variation that results from altered gene expression is usually irreversible (Karp 1995). Frequency of somaclonal alterations is a very important precondition for researchers working on tissue selection. To obtain a wider variability of important traits, it can be significantly enhanced by mutagenesis (Jain 1997b, 1997c). Orton (1987) reported a big number of gene mutation occurrences in cell culture, which could be up to 10^{-2} events per locus and even more. Jain (1993) observed various differences in a number of morphological and physiological traits in *Begonia* × *elatior* and *Saintpaulia ionantha* L. Development of tissue culture systems, using selective media for modulation of unfavourable environmental impacts, is very important for facilitating selection of plants possessing resistance/tolerance to environmental stressors (Bell *et al*. 1997; Butenko 1999; Jain 2001; Sowik *et al*. 2001; Singh *et al*. 2002; Biswas *et al*. 2009).

Nosov (1999) summarized data on somaclonal variability and selection of valuable somaclones obtained by numerous researchers, and concluded that cell and tissue selection for physiological traits did not guarantee availability of these traits in regenerated plants, even if they were conditioned by gene alterations. Processes of secondary metabolism, growth and development in a whole plant organism differ from those in cell suspensions. Nonetheless, when resistance/tolerance to an unfavourable environmental impact has a genetic basis and is driven by mechanisms operating within a cell, i.e. acts at the cellular level, the trait

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is considered to be stable. Most likely, a whole plant regenerated from such a resistant cell will exhibit the same resistance (Nosov 1999). Mechanisms driving traits such as tolerance to soil salinity, acidity and alkalinity (Dolgykh 1994), antibiotics, herbicides, analogs of amino-acids and nitrogenous bases, resistance to extreme temperatures (Popov *et al*. 1995) and pathogens affecting cells with phytotoxins (Dolgykh 1994) act at the cellular level. Therefore, tissue selection for these characters can be carried out *in vitro*.

Noteworthy results in cell and tissue selection for resistance to diseases and tolerance to salinity have been achieved in alfalfa (Frame *et al*. 1991), maize (Dolgykh 1994), potato (Behnke 1979), *Poncirus* (Beloualy and Bouharmont 1992), rice (Gregorio *et al*. 2002) and some other, mainly annuals and biennials species. Similar investigations have been carried out in strawberries, but the number or reports are not numerous. Tissue selection experiments for resistance to phytopathogenic fungi, such as *Botrytis cinerea* Pers., *Phytophthora cactorum* Leb. *et* Cohn (Schroet), *P. fragariae* Hickman and *Verticillium dahliae,* and for tolerance to salinity stress were undertaken in different countries (Maas *et al*. 1993; Solovykh and Tarabrin 1994; Orlando *et al*. 1997; Debnath and Teixeira da Silva 2007; Shaw *et al*. 2008; Biswas *et al*. 2009; Solovykh 2009), which resulted in finding this method to be useful and promising to improve individual traits.

P. cactorum causes severe damage to strawberry plants, such as crown rot and leather fruit rot. The disease is widespread in Russia (Novotelnova 1974; Drozdovsky and Barbatunova 1985). Yield losses in infected plants range from 50 to 100%, while infection of susceptible cultivars usually results in plant death. Grey mould, caused by *B. cinerea*, is one of the most destructive diseases attacking strawberries when environmental and cultural conditions are favourable for disease development. Pre- and post-harvest fruit rot is the main damage caused by this pathogen, which in wet conditions may lead to yield losses exceeding 50% in susceptible cultivars. Moreover, plant parts of especially vulnerable cultivars may also be attacked by the fungus (Scott and Lawrence 1975). The disease is particularly disastrous in regions with mild and moderately warm climates, abundant in rain falls. Mycelia of both fungi affect plant tissues with phytotoxins, killing them and subsequently developing on plant remains. Thus, resistance to either disease is likely dependent on cell tolerance to the toxins, which may allow selection for these traits *in vitro*, screening tissue cultures on selective media and using phytotoxins as selective agents.

In Russia, the necessity of strawberry lines tolerant to salinity is conditioned by the existence of wide cultivation areas of alkalescent and alkaline soils with excessive salt levels. Most strawberry cultivars are very sensitive to salinity (Ehling and Bernstein 1958; Dziadczyk *et al*. 2003). It affects plants unremittingly, breaks normality of physiological processes, deteriorates plant state and lowers their productivity (Udovenko 1977; Ashraf 1989; Ashraf and Ahmad 2000). More than a half of territories are supposed to get salinized by mid-century. Inappropriate agricultural practices and predicted effects of global climate changes will further deepen the problem (Yeo 1999). On the other hand, in some places only salty water is available for crop irrigation. However, the problem of soil and water salinization may be partially overcome by breeding and selection of new varieties.

A noteworthy progress in the study of and breeding for salinity tolerance and its related abiotic stresses has been reported in rice (Gregorio *et al*. 2002), which encourages analogous efforts in working with other species. Although reactions to salt stress in plant organisms are extremely complex, the advances in physiology, genetics and molecular biology achieved in some species, have greatly improved the understanding of plant responses to the stresses, such as changes of amino acid and protein contents in response to NaCl treatment (Hasegawa *et al*. 2000; Yokoi *et al*. 2002; Shin *et al*. 2004; Koiwa *et al*. 2006; Gao *et al*. 2007) and changes of reactive oxygen species (ROS) and antioxidant enzymes activity in root tip cells at the early stages of salt stress-induced programmed cell death (PCD), a rapid process which may help plants survive adverse stresses by eliminating cells, tissues or organs that render a plant more vulnerable to its environment (Li *et al*. 2007). Li *et al*. (2007) suggested that the response of root tips to salt should be divided into two phases: the salt stress responding phase and the PCD phase. In the responding phase, increased ROS induces scavenging-enzyme activities. This response removes toxic ROS and prevents PCD from occurring. If the stress prolongs, the cell antioxidant system is damaged, ROS will accumulate, overcome the threshold and initiate PCD events, such as mitochondria membrane permeability transition, the release of proteins from mitochondria intermembrane space, and finally lead to nuclear changes, such as chromatin condensation, nuclear deformation and DNA fragmentation.

Water containing up to 2,500 mg of chlorides is supposed to be successfully used for irrigation of salt resistant cultivars of many vegetables and some horticultural crops in desert regions (Dziadczyk *et al*. 2005). However, using soils with high salt contents requires selecting cultivars of agricultural crops, tolerant to salinity, because the soils cannot be irrigated with saline water without significant damage to plants (Choi *et al*. 2003; Li *et al*. 2007). In such circumstances, some biotechnological methods have certain chances to be helpful to solve the problem (Dziadczyk *et al*. 2003, 2005).

The first aim of this study was to assess tissue screening on selective media containing phytotoxins from *B. cinerea* and *P. cactorum* as a method for recovering strawberry lines, resistant to, using both pathogens. The other aim was to optimize the composition of selective media supplemented with NaCl for tissue screening to obtain strawberry lines tolerant to chloride salinity and to estimate different methods of testing the lines.

MATERIALS AND METHODS

Plant material and experimental design

In vitro selection for resistance to *B. cinerea* and *P. cactorum*, using callus tissues derived from 'Zolushka', a very high-yielding standard cultivar of mid-late term of fruit maturing, and 'Lvovskaya Rannyaya' (early ripening) was carried out in 2000–2003. Testing of selected lines, obtained during the study, along with promising somaclones of 'Urozhainaya CGL' (mid-season) selected earlier, for resistance to diseases was conducted in 2004.

Plants of 'Feierverk' (mid-season), 'Lvovskaya Rannyaya', 'Urozhainaya CGL' and 'Zolushka', all high-yielding and winter hardy in conditions of central Russia, were used as sources of explants to obtain callus cultures for tissue screening to select lines tolerant to salinity. All of them are typical June-bearing strawberry genotypes. The studies were performed in 2004–2007. Selected lines were tested for tolerance to the stressor in 2008.

Callus tissues of each cultivar on each variant of selective media were screened, using four successive passages (except for the screening for salt tolerance where only three passages were performed) and three replicates in each passage. Each replicate included 30 calli, therefore, 90 tissue specimens in total were grown in each passage, on each medium, at each percentage of any selective agent in the medium (three different concentrations of each selective agent were used for screening throughout) and in control (with no selective agents). Only calli exhibiting the most active growth and proliferation on selective media were selected for inclusion in each following passage.

Callus tissues proliferating on selective media were transferred to media for proliferation with no selective agents favouring cell growth and multiplication; thereafter fragments of obtained tissues were placed on selective media containing the same stressor again.

Calli, selected for resistance/tolerance to unfavourable factors, were used to regenerate adventitious shoots every two passages.

Leaves from the regenerated shoots were taken to induce callus formation. Then fragments of the latter, in their turn, were placed on appropriate selective media to carry on screening processes. Such an alternation of procedures allowed maintenance of a morphogenetic potential of selected tissues.

At the final stage of the tissue selection process, every callus line, selected for an exceptional tolerance rate exhibited in regard to whatever stressor, was used for regeneration of whole plants, and afterwards, regenerated plants were cloned to obtain sufficient plant material for further studies.

Induction of callus formation and proliferation

To initiate callus formation, strawberry leaf explants, taken from plants cultivated *in vitro*, were cultured on MS medium (Murashige and Skoog 1962) supplemented with either 2 mg l^{-1} 2,4-dichlorophenoxy-acetic acid (2,4-D) or IBA (all auxins, cytokinins and gibberellins were purchased from Sigma-Aldrich, Moscow, Russia). Explants were placed with the adaxial side in contact with the medium and then incubated under dark conditions at 25 ± 2°C. Callus formation was achieved in 30–40 days. Every 35–40th day calli were divided into fragments (subsequent calli), approximately 0.3 cm^3 each, which were transplanted onto a fresh prepared medium in separate vessels and cultured in the dark to induce callus regeneration.

Preparation of selective media for tissue screening

To conduct tissue screening for resistance to phytopathogens, selective media were prepared by adding fungal culture filtrates in media for callus subcultures. Filtrates of monthly fungal cultures, containing phytotoxins of *P. cactorum* and *B. cinerea*, were prepared in the Laboratory of Immunity of the Michurin Russian Research Institute of Genetics and Breeding of Fruit Plants. Efficient concentrations of the selective agents in selective media to be applied were determined experimentally. Cultural filtrates of phytotoxins from *P. cactorum* at 20% and those from *B. cinerea* at 15% (for 'Feierverk') or 20% (for 'Urozhainaya CGL' and 'Zolushka') restricted growth in 80–90% of calli (unpublished data). Hence, media containing filtrates of both fungi at 10, 20 and 30% were used for screening. Experiments in the Laboratory of Biotechnology of the Michurin Russian Research Institute of Genetics and Breeding of Fruit Plants proved that the thermal stability of phytotoxins from the pathogens was high enough to allow incorporating them with nutrient media prior to autoclaving (unpublished data), which was technically easier to perform.

Selective media for screening calli for tolerance to chloride salinity contained NaCl at 0.3, 0.6 and 0.9% (50, 100 and 150 mM, respectively).

Adventitious shoot regeneration, micropropagation and rooting of selected lines *in vitro*

Adventitious shoot regeneration from selected lines of calli which exhibited resistance/tolerance to the phytopathogens or salt toxicity, was made on MS and/or QL (Quoirin and Lepoivre 1977) media with macro- and micronutrients (both macrosalts of a first-grade purity and microsalts of the highest-grade purity were purchased from Reakhim, Shostka, Sumy district, Ukraine), supplemented with 2.0 mg l^{-1} BAP (98%), 0.5 mg l^{-1} IBA and 0.2 mg l^{-1} GA_3 (97%). Also, media without GA_3 and those supplemented with antioxidants, viz. ascorbic acid and reduced glutathione (95%, Sigma-Aldrich, Moscow, Russia), were applied to some selected lines in subtests. Regenerated shoots were micropropagated on a modified Boxus medium (Boxus 1974, 1992), viz. MS medium with 1.0 mg l^{-1} BAP, supplemented with 0.1 mg l^{-1} IBA, and rooted on half-strength MS medium (Yue *et al*. 1993) supplemented with 1.0 mg l^{-1} BAP and 0.5–1.0 mg l^{-1} IBA (depending on the cultivar used as tissue source). Additionally, the medium contained 20 g l^{-1} sucrose. For shoot micropropagation of 'Zolushka'-derived lines GA_3 at 0.5 mg l^{-1} instead of IBA was included in the medium.

In vitro plantlets were maintained at 22–24°C and a relative humidity within the range of 70–75%, under conditions of a 16-h photoperiod and a photosynthetic photon flux density (PPFD) from cool white fluorescent lamps ranging from 35 to 38 μmol m^{-2} s^{-1} (computed using tables of UKROP.info/TopTropicals.com).

Acclimatization of *in vitro*-rooted plants to *in vivo* conditions

A plastic-covered greenhouse equipped with trickle irrigation was used for acclimatization of *in vitro*-rooted plants to *in vivo* conditions. Only well-developed and rooted plants were chosen for transplanting. Before being transferred to the greenhouse, the plants were subjected for 9-10 days to a higher PPFD, beginning from a minimum of 70.0 μmol m^{-2} s^{-1}, which was gradually increased to 100.0 μmol m^{-2} s^{-1}, to stimulate plant photosynthetic activity. To acclimatize plants, the following routine procedures were performed: plants were extracted from vessels, washed with running tap water to eliminate the culture medium and planted on preliminary prepared beds in the greenhouse. A mixture of soil, peat and sand in equal proportions was used as the substrate. Acclimated plants were planted in the greenhouse in late May and early June. They grew in the greenhouse for 2.5 months, and later transferred to field conditions.

Preparation of inoculums and testing plants for resistance to phytopathogens

Testing plants for resistance to *B. cinerea* and *P. cactorum* was conducted using artificial leaf inoculation with spore-mycelium suspensions of the fungi (mixes of mycelium fragments and conidia of *B. cinerea*, and mycelium, sporangia and zoospores of *P. cactorum*), followed by incubation of inoculated leaves in Petri dishes at 100% air humidity.

Inoculums were prepared by homogenization of a gelatinized wort-based medium, used for fungi cultivation, along with either fungal culture. A medium containing 10 g agar and 100 g wort per l of water was prepared, followed by cultivation of a fungal culture in a test tube (to enlarge a medium surface area and obtain a larger fungal population, test tubes with a hot medium poured into them were disposed at 15° to the table surface until the medium was set) in a thermostat at 23 ± 2°C over 3 weeks. Thereafter, contents of the test tube were stirred up, thoroughly homogenized and diluted with distilled water to a final volume of 100 ml. Afterward, mixtures were additionally diluted to adjust to approximately 30,000 conidia per ml for *B. cinerea* and 12,000–15,000 sporangia per ml for *P. cactorum*, and used for leaf inoculation.

Ten completely developed leaves of each line were used to estimate resistance to each disease. Leaves were immersed in an inoculum and placed over blotting paper moistened with distilled water in Petri dishes. The inoculated leaves were incubated in the closed Petri dishes at 25 ± 2°C. As soon as blotting paper became drying out, it was carefully moistened again to maintain 100% relative humidity inside the Petri dish.

Symptoms of disease development were usually observed in 5–7 days after inoculation with both fungal mixtures. Disease symptoms were estimated using a 6-point scale, where 0 = no symptoms, line is resistant; 1 = weak symptoms that appeared on less than 10% of leaf blade surface, line is tolerant; 2 = disease symptoms appeared on 10–25% of leaf surface, line is relatively tolerant; 3 = 25–50% of leaf blade surface is affected with disease, line exhibits low tolerance; 4 = 50–75% of leaf surface with disease symptoms, line is susceptible and 5 = leaves fully brown in colour and dead. This scale has been accepted in Russia for the evaluation of most economically important fungal strawberry diseases in field conditions (Shokaeva and Zubov 1999).

Testing for tolerance to salinity

1. In vitro testing

Preliminary testing of *in vitro*-micropropagated plants from selected lines for tolerance to chloride salinity was conducted on micropropagation culture medium supplemented with 0, 50 or 100 mM NaCl. MS medium, containing 30 g l^{-1} sucrose and supplemented with 1 mg l^{-1} BAP, 0.1 mg l^{-1} IBA and 1 mg l^{-1} GA_3, was used for this purpose. Cultures were maintained at 23–25°C under a 16-h photoperiod and a PPFD from fluorescent lamps ranging from

40.0 to 45.0 μmol m^{-2} s^{-1} for 35-45 days depending on line and level of salt tolerance. Shoot growth, proliferation rate and percentage of leaf death caused by salt toxicity were measured. A 6-point scale was used for tolerance estimation. Plantlets developed as those in control were characterized as resistant to salt stress (score 0). Lines showing an average decline in plant development lower than 10% were tolerant to salinity and ranked with score 1. Score 2 was given when decline in growth and development did not exceed 25%, indicating relative tolerance. If growth was slow, while damage to plants ranged from 25 to 50%, line was given score 3, which was evidence of its low tolerance. Almost no growth resulted in score 4, while score 5 designated plant death.

To avoid unnecessary costs, only the plantlets that exhibited a significantly higher tolerance compared with that of initial cultivars, were subsequently rooted, acclimatized in the greenhouse and planted in the field.

2. Laser analysis of tissue microstructure

The method of laser analysis of leaf tissue microstructure (LAM), using a device named FSR–03–08 (Budagovsky *et al*. 1998, 2007), was employed to test plants growing in field conditions for resistance to salt toxicity. The device has been developed in the Michurin Research Institute of Horticulture (Engineering Centre, Michurinsk, Tambov District, Russia) by Budagovskaya ON (Budagovskaya 2004). It consists of a measuring module, a power block and a digital tester (10 × 15 cm). Additionally to this, a stop-watch is needed to conduct measurements. The method is based on a connection of a functional state of chlorophyll-containing plant tissues to dynamics of amplitude-phase characteristics of laser rays, emanated by the device and diffused with the tested plant tissues (Budagovskaya *et al*. 2007).

Ten discs, 1.5 cm in diameter, obtained from leaf blades of each selected line under study, were dipped in Petri dishes half-filled with distilled water (control) or a sodium chloride solution at 1.2% (200 mM). The functional state of leaf tissues was determined by measuring diffused light intensity with 'FSR–03–08' at 24, 48 and 72 h after beginning the treatments. Each leaf disc was positioned in the working sector of the measuring module with the adaxial side up, after which a helium-neon laser with a wavelength of 632.8 nm was lighted up. Diffused light intensity was measured twice, at the beginning of irradiation and 60 s later. The period was chosen as optimal in previous experiments (unpublished data). An index of functional state (IFS) of plant tissues, depending on capabilities of chlorophyll-protein complexes to perform light-induced re-organizing themselves in conformity with changed conditions, was computed using obtained data and the following formula:

$$IFS = (I_{max} - I_{min})/I_{min},$$

where IFS = index of functional state, I_{max} = diffused light intensity at the beginning of irradiation and I_{min} = diffused light intensity in 60 s.

3. Leaf diagnostics

Newly developed full-sized leaves, ranging by area from a minimum of 70 cm^2 in 'Zolushka' and its derivatives to a maximum of 110 cm^2 in 'Lvovskaya Rannyaya', were taken from each selected line (ten replicas per line) and incubated in a test tube with their petioles immersed in 1.2% NaCl solution (200 mM). Necroses that emerged on the leaf blades, being caused by salt toxicity, were measured in 48 and 72 h, using the following 6-point scale: 0 = no necroses, line is resistant to salinity; 1 = necroses emerged on a maximum of 10% of leaf surface, line is salt-tolerant; 2 = 10–25% of leaf surface is spotted with necroses, line is relatively tolerant; 3 = 25–50% of leaf surface is necrosis-diseased, line is rather susceptible; 4 = 50–75% of leaf surface is necrosis-diseased, line is very susceptible and 5 = leaves are dead.

In some leaves, salt damages manifested as green dry areas instead of necroses areas.

32 lines in total were tested: eight lines derived from 'Zolushka', four from 'Feierverk', eight from 'Lvovskaya Rannyaya' and twelve lines obtained from 'Urozhainaya CGL'. Additionally to them, all the control cultivars were tested for salt tolerance using this method.

Morphological and agricultural characterization of selected lines

In the MV2 and MV3 generations, all selected lines were examined for stability of the characters which they were selected for. The plants that exhibited their instability were destroyed. High fertility of flowers was the other trait that should be unconditionally characteristic of them.

Morphological characterization and agricultural evaluation of selected strawberry lines were performed using UPOV system (Shokaeva and Zubov 1999; Shokaeva 2006, 2008). Yield and its components, and fruit size and quality were the main characters evaluated.

Statistical analyses of data

Statistical analyses have been performed, using a package of statistics, based on or analogous to ANOVA tests developed in the SAS Institute (Version 6, MathSoft, Inc., USA), and adapted to a Microsoft Office Excel Software. Means, significance of differences between means at $P \leq 0.05$ and standard errors were computed for each study. Data from tests for both tolerance to phytotoxins and salt tolerance *in vitro* and data obtained from LAM analysis were statistically analysed as bifactorial experiments, where effects of main factors were studied to compute related LSD_{05} values. These factors were as follows: for tests *in vitro* A = medium variant and B = passage, and for LAM analysis A = variant of treatment of leaf discs and B = selected line. Results of tests for resistance to diseases using leaf inoculation, and tests for salt tolerance using leaf diagnostics were analysed using Duncan's multiple range test (available in the statistics package mentioned above).

RESULTS AND DISCUSSION

Effects of callus tissue screening for resistance/tolerance to biotic and abiotic stressors

An alternation of calli screening and conditions of subculture for selected tissues, which favoured cell growth and multiplication, resulted in obtaining rather stable tissue lines, visibly tolerant to phytotoxins from *B. cinerea* and *P. cactorum* (**Fig. 1**). Amounts of callus tissues derived from 'Zolushka', which remained capable to proliferate on media containing phytotoxins of *B. cinerea*, became evidently higher in every following passage, although differences were frequently insignificant (**Fig. 2**). This was particularly noticeable in the 20% cultural filtrate treatment. Increment in tissue tolerance to *P. cactorum* was less perceptible (**Fig. 3**). As expected, few cells were able to withstand the highest phytotoxin concentration. Somewhat lower percentages of tolerant tissues were obtained in 'Lvovskaya Rannyaya' (data not shown). These data corroborated that obtained earlier in an experiment carried out in 1997–2000, where tissues from 'Feierverk' and 'Urozhainaya CGL' were screened. The results were especially prominent in variants with tissues derived from 'Urozhainaya CGL' (Solovykh and Tarabrin 2004). In this cultivar, screening on selective media containing fungal filtrates with phytotoxins from *P. cactorum* at 20% yielded a 33.3% of tolerant calli in passage 4 vs. 9.7% in passage 1. Screening for resistance to *B. cinerea* on media containing cultural filtrates at 20% led to 5.9% of proliferating calli in passage 1, whereas their proportion reached 30.0% in passage 4. 'Lvovskaya Rannyaya' gave lower percentages of calli tolerant to the pathogens. To all appearance, plant tissues derived from the cultivar are more susceptible to the diseases. Susceptibility to *B. cinerea* and *P. cactorum* distinguishes many early-season June-bearing strawberry genotypes (pers. obs.).

Similar phenomena were observed when calli were screened for tolerance to salinity. Proportions of calli proliferating under the highest NaCl concentration increased from passage to passage, as can be seen in variants with tis-

Fig. 1 Callus growth and proliferation on MS medium containing cultural filtrate of *P. cactorum* at 20%. Callus tissues are derived from 'Zolushka'. Data correspond to passage 3 after 35 days of cultivation.

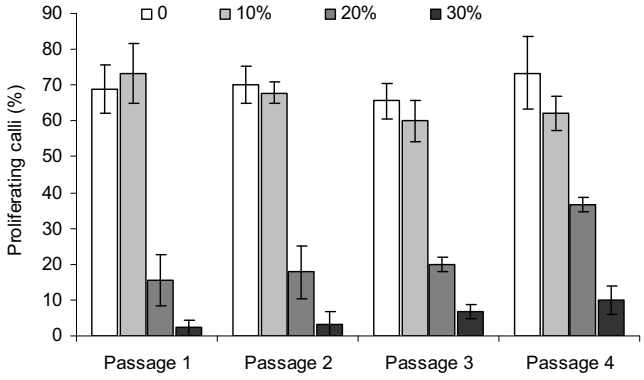

Fig. 2 Proliferation of calli derived from 'Zolushka' on MS medium with toxins of *B. cinerea*. Variants are: without (0.0%, control) and with cultural filtrates of phytotoxins at 10, 20 and 30%. Values represent mean ± standard error (SE). LSD^{A}_{05} = 52.9, LSD^{B}_{05} = 15.1, where A = medium variant, B = passage.

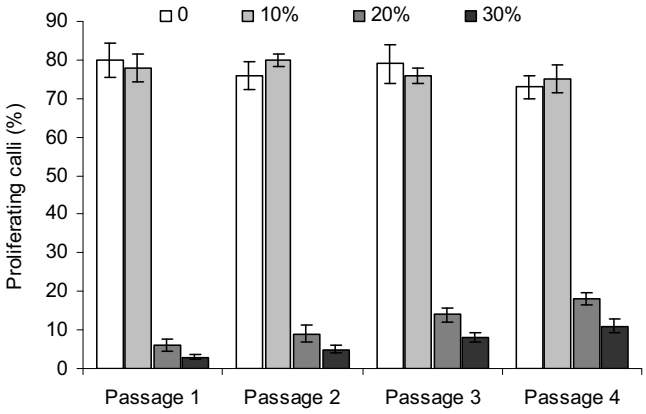

Fig. 3 Proliferation of calli derived from 'Zolushka' on MS medium with toxins of *P. cactorum*. Variants are following: without (0.0%, control) and with cultural filtrates at 10, 20 and 30%. Values represent mean ± standard error (SE). LSD^{A}_{05} = 57.2, LSD^{B}_{05} = 8.3, where A = medium variant, B = passage.

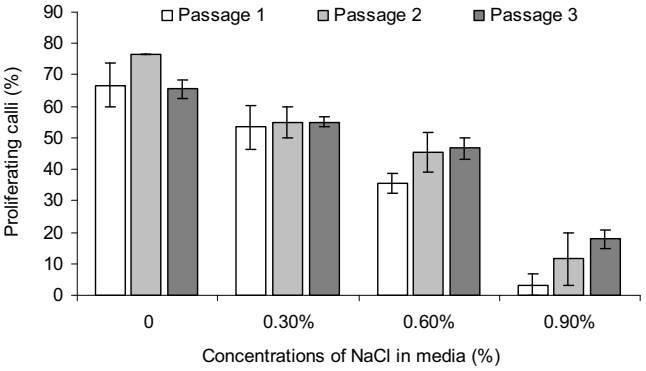

Fig. 4 Numbers of calli that kept proliferating on MS medium supplemented with NaCl. Variants are: without (0.0%, control) and with NaCl at 0.3, 0.6 and 0.9%. Calli are derived from 'Zolushka'. Values represent mean ± standard error (SE). LSD^{A}_{05} = 29.8, LSD^{B}_{05} = 10.9, where A = medium variant, B = passage.

sues from 'Zolushka' (**Fig. 4**). Close percentages of tolerant tissues were obtained when calli, derived from 'Feierverk' and 'Urozhainaya CGL', were screened (data not shown). In the case of tissues derived from 'Lvovskaya Rannyaya', proportions of salt tolerant lines were lower. In passage 1, only 6.7% of calli demonstrated capability to proliferate on a medium containing NaCl at 0.6%, while the amount of tolerant tissues in passage 3 at the same salt concentration reached 21.7%. It is likely that plant tissue cells of the genotype exhibit a higher water requirement per g of raw weight, typical of most cultivars fruit of which mature early (Udovenko 1977), which can more rapidly result in toxic levels of ion accumulation. Influx of Na^{+} negatively impacts intracellular K^{+} influx, attenuating acquisition of this essential nutrient by cells, alters the H^{+} electrochemical gradient and dissipates the membrane potential, thereby facilitating uptake of Cl^{-} (Hasegawa *et al*. 2000). This might to some extent elucidate the lower salt tolerance of the calli *in vitro*. Proportions of calli that kept growing and proliferating on media containing NaCl at 0.9% (stress treatments) were very low compared with the other variants, particularly of those derived from 'Lvovskaya Rannyaya', although gradually increased, reaching the highest percentages in passage 3 (**Fig. 4**, data on the other cultivars not shown). Calli that proliferated actively on media with high NaCl concentrations could form plants possessing genetically conditioned tolerance to this destabilizing factor. To test this hypothesis, these calli were cultured on regeneration medium to induce adventitious shoot regeneration.

Getting over difficulties of adventitious shoot regeneration and micropropagation

Adventitious shoot regeneration from the selected calli was the following critical stage of this work. Strawberry callus tissues are deemed to have good abilities for plant regeneration. However, tissue selection requires subculture of callus for a long time (up to 14 months) on artificial cultural media, after which shoot regeneration from the cells becomes a considerable problem. For instance, average morphogenesis frequency from calli derived from 'Lvovskaya Rannyaya' after 12-month subculture (eight passages) decreased to 3.6%, from calli of 'Urozhainaya CGL' and 'Zolushka' to 1.5 and 1.2%, respectively, and no regeneration occurrence was observed in the case of callus tissues obtained from 'Feierverk'. After 6-month subculture adventitious shoots could be regenerated from 6.2% of 'Feierverk'-derived calli, 8.5% of calli from 'Lvovskaya Rannyaya' and 9.9% of calli from either of the two other cultivars. Regeneration frequency after two cultivations (three months) on selective media ranged from 24.0 to 51.4%. To facilitate solving this problem, a callus subculture on a selective medium was alternated with adventitious shoot regeneration from the callus tissues. There is usually some probability of obtaining resistant plants from a callus tissue, selected in a single subculture on a selective medium, however, due to

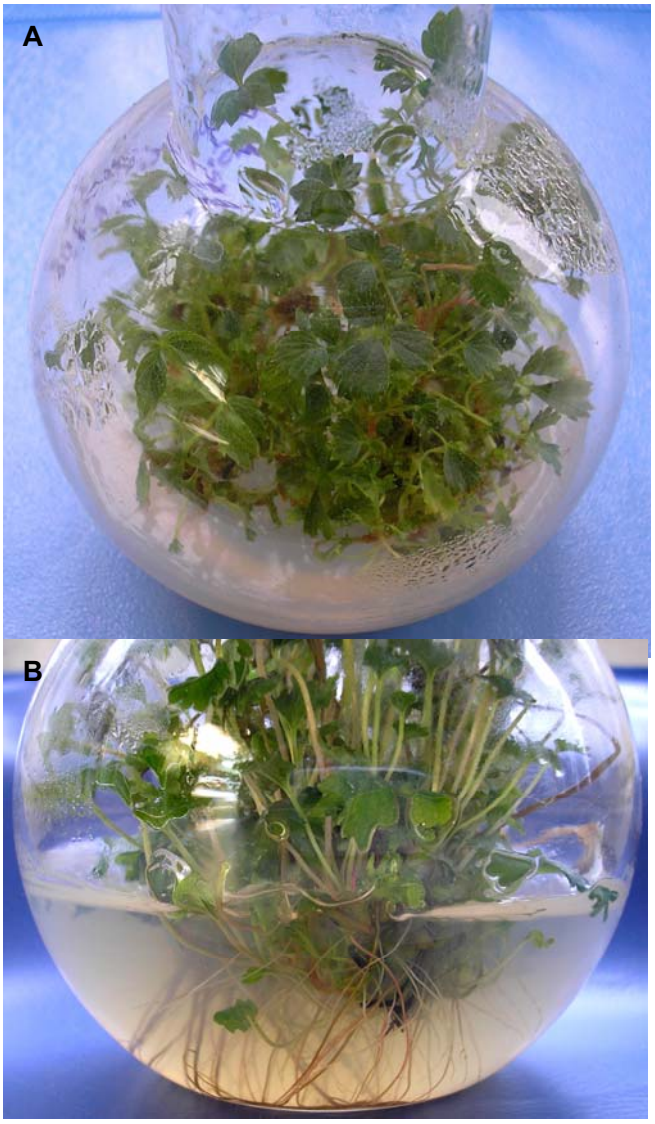

Fig. 5 (A) Micropropagation and (B) rooting of salt-tolerant plants *in vitro* on MS medium. Media are supplemented with: (**A**) 1.0 mg l^{-1} BAP and 0.1 mg l^{-1} IBA, 36th day of cultivation; (**B**) 1.0 mg l^{-1} BAP and 1 mg l^{-1} IBA, 40th day of cultivation. Plants are regenerated from a callus line derived from 'Zolushka'.

heterogeneity of callus cells, there is no guarantee that morphogenesis will occur exactly from those resistant cells. Only two of eight plants, regenerated from calli selected after two first passages on a selective medium that contained cultural filtrate of *P. cactorum* at 20%, turned out to be resistant to the pathogen itself. Amounts of resistant cells, as well as the number of resistant plants regenerated from them, increase from passage to passage thanks to a sustaining screening process, but when cultivation *in vitro* lasts seven or eight passages, declining of a morphogenetic potential of callus tissues becomes irreversible. Quantities of plants, regenerated from 'Zolushka'-derived calli that were selected after four passages of subculture on selective media and exhibited resistance/tolerance to different stressors, amounted to 43.8-85.0%. 54.5-61.5% of resistant/tolerant plants were obtained from 'Lvovskaya Rannyaya', 28.6-80.0% from 'Urozhainaya CGL' and 50% from 'Feierverk'. On the other hand, the *in vitro* tissue culture frequently led to accumulation of somaclonal changes (pers. obs.), including those affecting useful and economically important traits of initial cultivars. Hence, concluding stages of tissue selection must be accompanied by field testing of selected plants.

MS medium was most suitable for regeneration of strawberry plants. Optimal ratio of auxins and cytokinins in a medium is dependent on an endogenous balance of phytohormones in each particular genotype (Rastorguyev 1996). The strawberry cultivars used in this study differ in regeneration frequency on media of different hormonal compositions (unpublished data), nevertheless, calli of most cultivars provided intensive shoot regeneration on the medium supplemented with 2 mg l^{-1} BAP and 0.5 mg l^{-1} IBA. As previously observed in disease resistant and tolerant lines derived from regenerated calli of 'Urozhainaya CGL' and 'Feierverk' (Solovykh and Tarabrin 2004), 0.2 mg l^{-1} GA_3 positively influenced the regeneration process, favouring shoot growth, although differences were often insignificant (data not shown). Antioxidants, viz. 250–750 μM ascorbic acid and 100−250 μM reduced glutathione, also favoured shoot regeneration, increasing vigor of plantlets, although they usually had no significant influence on either shoot number or shoot length (data not shown). The highest regeneration frequency of plants with the characters which calli were selected for, achieved in optimal variants, fluctuated from 6% ('Lvovskaya Rannyaya') to 11% ('Urozhainaya CGL').

Fig. 6 Salt-tolerant strawberry plants, acclimatized to growing in field conditions. Lines are derived from 'Zolushka' and obtained using tissue selection.

As proposed by Boxus (1974, 1992), adventitious shoots were micropropagated on MS medium supplemented with 1.0 mg l^{-1} BAP. Although micropropagation of strawberry cultivars *in vitro* may be successfully carried out without auxins, the addition of 0.1−0.2 mg l^{-1} IBA has been found to favour shoot development (Muratova *et al*. 2004). A combination of 1.0 mg l^{-1} BAP and 0.1 mg l^{-1} IBA was optimal for micropropagation of most selected lines (**Fig. 5A**). In lines derived from 'Zolushka', using proliferation media with 0.5 mg l^{-1} GA_3 instead of IBA resulted in a somewhat larger shoot size in approximately 50% of the lines. Although when cultivated for a long period of time, strawberry plantlets are able to root even without auxins, media that contained 1 mg l^{-1} IBA turned out to be particularly felicitous for *in vitro* rooting of selected lines, favouring both quick root formation, strength and well-balanced development of plantlets (**Fig. 5B**).

Results of testing for resistance/tolerance to the stressors and main achievements of the selecting work

Depending on selected lines and initial cultivars, 69 to 85% of plantlets rooted *in vitro* were successfully acclimatized to *in vivo* conditions and subsequently planted in the field (**Fig. 6**). Tests for resistance to *P. cactorum*, using leaf inoculation method which exhibited in similar studies on grape cultivars being precise enough to be used instead of crown inoculation (Shtin 1971), proved most selected lines to be resistant or more tolerant to the fungal infection compared

Table 1 Results of testing for resistance to *B. cinerea* and *P. cactorum* using artificial leaf inoculation.

Initial cultivar	Percentage of leaves/lines with scores 0 and 1	
	B. cinerea	***P. cactorum***
Z (control)	0.0	5.0
Z (selected lines)	43.8	83.7
LR (control)	0.0	2.2
LR (selected lines)	54.5	66.7
U (selected lines)	28.6	80.0

Z: Zolushka, LR: Lvovskaya Rannyaya, U: Urozhainaya CGL

with initial cultivars. In four days following inoculation, development of the disease on most leaves, taken from 'Lvovskaya Rannyaya' and 'Zolushka' (control), was scored with 3, 4 and 5; leaves from 'Lvovskaya Rannyaya' had particularly heavy symptoms. The remaining leaves were almost all given score 2. Such a high susceptibility was characteristic of the cultivars in wet conditions favouring the disease development, while most leaves, taken from the selected lines of both cultivars, exhibited resistance or tolerance (scores 0 and 1) to the disease (**Table 1**), and only a few leaves were given score 3. Percentage of lines, resistant or tolerant to *B. cinerea*, was lower, particularly among somaclones of 'Urozhainaya CGL'. At the same time, none of the leaves that were taken from control plants, had scores 0, 1 or 2. In the preceding study (1997–2000), where 'Urozhainaya CGL' (Solovykh and Tarabrin 2004) and 'Feierverk' (unpublished data) were used for tissue selection, 52.4% of selected lines derived from 'Urozhainaya CGL' demonstrated resistance/tolerance to *B. cinerea* vs. 0.0% of resistant leaves in control. 56.7% of lines derived from 'Feierverk' also revealed tolerance compared with 2.1% of leaves taken from the cultivar. 64.8% of somaclones of 'Urozhainaya CGL', selected for tolerance to toxins of *P. cactorum*, proved being resistant or tolerant to the fungus. Proportion of equally resistant/tolerant lines derived from 'Feierverk' amounted to 81.7%. Taking into account the fact that the conventional method of breeding and selection for resistance to *P. cactorum* is usually significantly less resulting (Eikemo *et al*. 2003; Shaw *et al*. 2008), the method of tissue selection can be one of the vital tools to gain the desirable feature.

Some lines, selected for tolerance to phytotoxins from *B. cinerea* or *P. cactorum*, revealed neither resistance nor tolerance to the phytopathogens themselves, as this can be seen from **Tables 1** and **2**. Apart from these studies, a number of researchers (Behnke 1979; Orton 1987; Frame *et al*. 1991) obtained plants, regenerated from cells or calli tolerant to phytopathogen toxins, which were resistant or tolerant to the phytopathogen itself. At least it was true in regard to *P. cactorum* (Solovykh and Tarabrin 2004). In other words, possessing a tolerance to toxins at the cellular level often conditioned a resistance/tolerance of the regenerated plant to the disease (Nosov 1999). However, exceptions might occur when defensive mechanisms of plant cells were based on hypersensitive responses (HRs) to the fungus penetration. Also, resistance happens in some cases to be epigenetic. Besides, heterogeneity of callus cells, which is kept even after long cultivation terms on selective media, might play its role, and cells that experienced a somewhat less tight pressure of a selection tool and possessed a poorer tolerance, could participate in plant regeneration instead of tolerant ones, producing less tolerant plantlets than that might be expected.

Results, obtained in tissue selection for resistance to *B. cinerea*, bear evidence that tolerance to phytotoxins from the fungus does not ensure resistance to the pathogen in each case. It was especially clear in the study where clones of 'Urozhainaya CGL' were tested (**Table 1**). Nevertheless, in view of the fact that considerable proportions of resistant and tolerant somaclones were selected, one could say that this particular method of tissue selection is possible to be a success. This method may be an important addition to the conventional method of breeding for the character and gaining recognition transgenic methods enhancing resistance to the important disease in strawberry genotypes (Jain and Pehu 1992; Vellicce *et al*. 2006).

Results, obtained during evaluation of selected lines, allow concluding that the method, based on leaf inoculation with spore-mycelium suspensions, has proved its reliability and may be used for estimation of strawberry plant resistance to the diseases. It requires neither much time nor large costs, which is its most important advantage compared with other methods, used in greenhouses and field conditions, with crown inoculation methods in particular (Bell *et al*. 1997; Parikka 2007).

Preliminary testing of selected lines for tolerance to salinity *in vitro* was combined with micropropagation of shoots, regenerated from selected calli. The tests exhibited that selected lines, although differed from each other in tolerance, were still superior to initial cultivars. Whereas plantlets of the latter were usually affected by salt toxicity heavily (**Fig. 7A**), most selected lines demonstrated visibly higher capabilities to withstand the stressor (**Fig. 7B**), although when salt levels in media were high, they were visibly depressed compared with control plants (**Fig. 7C** corresponds with selected line 3-1-07 growing in the absence of NaCl). Salinity slowed down shoot growth, led to sinking of the propagation coefficient, caused leaf necroses and partial leaf death to practically all selected lines (data not shown).

In saline conditions, to protect actively growing and metabolizing cells, plants regulate ion movement into tissues (Flowers and Yeo 1992; Munns 1993). One mode by which plants control salt flux to the shoot is regulating the entry of ions into the xylem stream. The other, the accumulation of large quantities of ions in mature and old leaves, which then dehisce, has often been observed under salt stress (Flowers and Yeo 1992; Munns 1993). In a function as ion sinks, old leaves may restrict ion deposition into meristematic and actively growing and photosynthesizing cells. An alternative possibility is that cellular ion discrimination is a natural consequence of transpirational and expansive growth fluxes, cell morphology, and degree of intercellular connection. Meristematic cells, which are not directly connected to the vasculature, are less exposed to ions delivered through the transpiration stream, and their small vacuolar space is not conducive to ion storage. *De facto*, the solute content of tissues containing cells with little vacuolation (e.g. meristematic regions) is predominated by organic osmolytes and in tissues with highly vacuolated cells by ions (Wyn Jones 1981; Binzel *et al*. 1988). Judging overall, tolerant plants have higher capabilities to control contents of the streams and protect the most actively growing and photosynthesizing tissues and organs by syn-

Table 2 Quantities of lines, resistant to *B. cinerea* and *P. cactorum*, obtained using tissue selection.

Initial cultivar	Phytopatogen	Lines showing resistance after leaf inoculation	Lines showing no symptoms in field conditions	Resistant/tolerant lines producing fruit not inferior to initial cultivar
Z	*B. cinerea*	7	7	4
Z	*P. cactorum*	17	17	9
LR	*B. cinerea*	6	4	0
LR	*P. cactorum*	8	6	2
U	*B. cinerea*	2	2	2
U	*P. cactorum*	16	14	10

Z: Zolushka, LR: Lvovskaya Rannyaya, U: Urozhainaya CGL

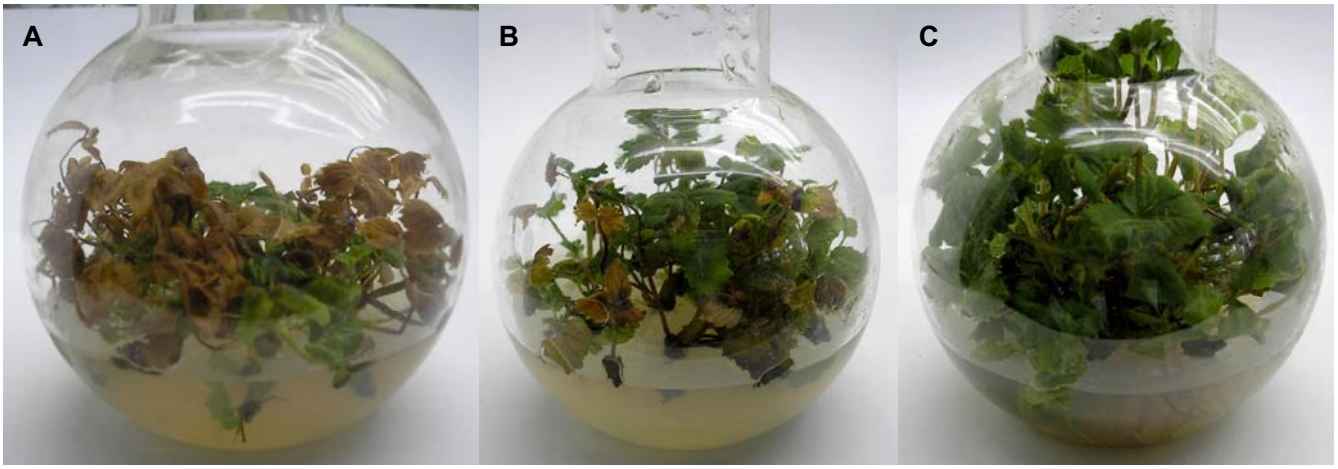

Fig. 7 Growth and development of *in vitro* plants on MS medium: (**A-B**) containing NaCl at 0.6% (100 mM); (**A**) 'Zolushka' (control). (**B**) Line 3-1-07 derived from 'Zolushka'. (**C**) Line 3-1-07 on MS medium without NaCl. 38th day of cultivation in all variants.

Table 3 Quantities of lines, tolerant to chloride salinity, obtained using tissue selection and tested with different methods.

Initial cultivar	Lines regenerated from calli	Lines showing tolerance being tested *in vitro*	Lines with tolerance proved by LAM tests	Large-fruited lines proved to be tolerant by leaf diagnostics
Z	12	8	6	3
F	8	4	4	0
LR	13	8	7	4
U	18	12	10	7

Z: Zolushka, F: Feierverk, LR: Lvovskaya Rannyaya, U: Urozhainaya CGL

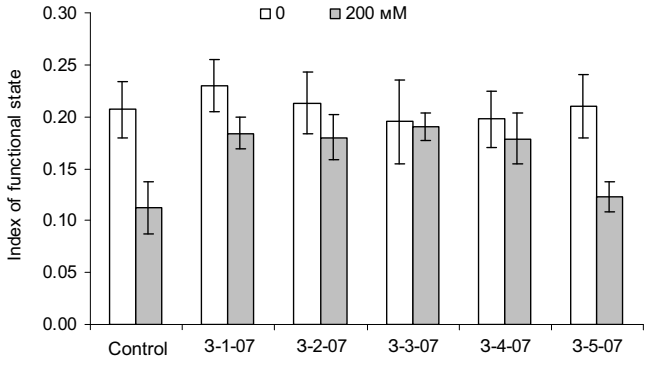

Fig. 8 Indices of functional state of salt-tolerant lines, determined with LAM method. Measuring was carried out after 72-h treatments with distilled water (0, control) and solution of NaCl at 1.2% (200 mM). Lines are derivatives from 'Zolushka', obtained using tissue selection. Values represent mean ± standard error (SE). LSD^{A}_{05} = 0.07, LSD^{B}_{05} = 0.05, where A = variant of treatment, B = line.

thesis of special compounds regulating biochemical pathways. Hasegawa *et al*. (2000) have defined determinants of salt stress tolerance as effector molecules (metabolites, proteins, or components of biochemical pathways) that lead to adaptation and as regulatory molecules (signal transduction pathway components) that control the amount and timing of these effector molecules. Stress adaptation effectors are categorized as those that mediate ion homeostasis, osmolyte biosynthesis, toxic radical scavenging, water transport, and transducers of long-distance response coordination (Rhodes and Samaras 1994; Niu *et al*. 1995; Asada 1999).

Testing selected lines, grown in field conditions, using the LAM method, revealed 84.4% of somaclones to possess an elevated tolerance to the salinity stress compared with initial cultivars (**Fig. 8**; **Table 3**), although lines, similar to the control, also occurred, for instance, line 3–5–07 derived from 'Zolushka' (**Fig. 8**). Comparable data were obtained when somaclones of the other cultivars and control plants were tested using the method. For example, leaf tissues of a selected line derived from 'Urozhainaya CGL', U 7-07, showed IFS = 0.24 after 24-h soaking in 200-mM NaCl solution, 0.23 after 48-h and 0.21 after 72-h treatment, whereas corresponding IFS values in the control cultivar were 0.20, 0.14 and 0.12, respectively. Computation of IFS values for plant tissues, taken from 'Lvovskaya Rannyaya', found out a noticeable decrease of its average value after 72-h soaking in NaCl solution by 32.7% compared with the control treatment with distilled water, while the difference in selected lines did not exceed 8% (Solovykh 2009). To be compared with the results obtained in the experiment with soaked leaf discs, measuring IFS of tissues taken from leaves of 'Lvovskaya Rannyaya' that were incubated in test tubes with their petioles immersed in 1.2% (200 mM) NaCl solution, using the same method, led to comparable results: IFS = 0.20 after both 24-h and 48-h treatments, 0.16 in 72 h, and 0.10 in 96 h, while values of this index for a selected line (LR 6-07) derived from the cultivar, were 0.23, 0.22 and 0.21, respectively (Solovykh 2009).

This method has proved being somewhat more sensitive (Solovykh *et al*. 2009) than the method of pulse amplitude modulation (PAM) of chlorophyll fluorescence, proposed by Schreiber *et al*. (1986), which has been used in last decades for different purposes, first of all for estimation of photosynthetic activity and photoinhibition in leaves (Krause and Weis 1991; Lutts *et al*. 1996; Goh *et al*. 1999; Solovykh *et al*. 2009). Also, 'FSR–03–08' can be successfully employed to determine chlorophyll contents in leaves and other chlorophyll-containing tissues by computation of a coefficient of permeability for rays of the red spectrum, using data obtained by measuring with the device.

Studies of chlorophyll characteristics, performed in rice under salt stress conditions, resulted in the finding that salinity alters chloroplast ultrastructure, causing a prominent swelling of thylakoids (Yamane *et al*. 2003, 2004). The ultrastructural changes were induced by ion toxicity or ionic imbalance (Yamane *et al*. 2003), while salt-induced oxidative stress was responsible for the alteration of thylakoids membrane property (Yamane *et al*. 2004). A few years later, evaluating rice seedlings treated with NaCl solution of different concentrations, these researchers detected chlorophyll fluorescence emission with a PAM instrument and examined for chloroplast ultrastructure in the regions where chlorophyll fluorescence had been recorded (Yamane *et al*. 2008). NaCl treatment caused swelling thylakoids, which

was quantified by the percentage of the length of swollen thylakoids to the total length of them. This value increased with increasing of NaCl concentration. A minimal fluorescence yield (after treatment with nutrient solution without NaCl) was not increased by treatments with 75 or 100 mM NaCl. On the other hand, the index increased and the swelling of thylakoids was prominent at 150 and 200-mM NaCl treatments. A high concentration of NaCl (200 mM) caused an increase in fluorescence yield within three days. An increase of fluorescence yield is characteristic of destruction of photosynthesis II reaction centres or inhibition of transfer of excitation energy from the antennae to the reaction centres. The highest level is theoretically the fluorescence emission when all reaction centres are open and the photochemical quenching is maximal. These results suggested that the increase in fluorescence yield index under salt stress was correlated with the ultrastructural damage (Yamane *et al*. 2008). Similar phenomena were observed in NaCl-treated maize (Hasan *et al*. 2006). The swelling of thylakoids under salinity is induced by lipid peroxidation caused by ROS, such as H_2O_2 and ·OH derived from H_2O_2 (Yamane *et al*. 2004), which are also related to the destruction of reaction centres.

The speculation suggests that a similar ultrastructural distortion of chloroplasts might take place in strawberry leaf tissues and be the reason of higher indices of light diffusion, measured with 'FSR–03–08' after their treatment with 200-mM NaCl solution compared to those treated with distilled water, and, accordingly, of the lower IFS values computed using the indices of diffused light intensity. The fact that this sinking of IFS values was no more than slight after 24-h treatment with the saline and prominent in most cases after 72-h soaking in it is additional evidence to allow the supposition of conformity of the phenomena to the results obtained in the photochemical and chloroplast structure investigations performed on rice and maize.

Leaf diagnostics that was employed to estimate salinity tolerance of the most promising strawberry lines in the field had much in common with the method usually used for classification of rice cultivars for salt tolerance by visual score at seedling stage, when seedlings, grown until 3rd to 4th leaf stages, then were treated with a nutrient solution containing 0.5% NaCl by bottom watering (Lee *et al*. 2003). The difference was that in this research only leaves were taken for the test instead of small intact plantlets, and a higher concentration of NaCl solution was used for leaf incubation. Herewith the protecting function of roots (Hasegawa *et al*. 2000; Li *et al*. 2007) was removed, and tissues underwent the stress treatment directly. It was a matter of a few days to find out which lines could withstand the severe salinity stress and to what extent. The visual score based on necrosis ratio appeared to be a rapid, simple and fairly reliable assessment tool, based on which, the lines were given definitive estimates in accordance with their salt tolerance level.

So far selected lines have not yet been completely evaluated and described. Some morphological and physiological traits, first of all characters contributing to fruit quality, winter hardiness and frost resistance of flowers are to be measured and estimated to be compared with those of the cultivars which they were derived from. Also, possible undesirable traits are needed to be found out.

Long cultivation of calli on nutrient media led to accumulation of somaclonal alterations touching on different useful traits of the cultivars used in the studies, some of which would be of interest for both strawberry fruit growers and researchers, because might be used for studying regularities of emergence and variability of different somaclonal alterations. Several lines, derived from 'Zolushka' and superior to the cultivar in resistance to fungal diseases, differed from it in inflorescence count per plant, flower count per inflorescence, fruit size and runner formation. Also, lines of 'Lvovskaya Rannyaya', 'Urozhainaya CGL' and 'Zolushka' with noticeably differing terms of fruit maturing were selected. Fruit of two somaclones of 'Lvovskaya Rannyaya' began to ripen 5-7 days later. One selected line descending from 'Zolushka' revealed a similar tendency, while a clone of 'Urozhainaya CGL' produced fruit that started to mature a week earlier compared with the cultivar. A line, derived from 'Zolushka', selected for salt tolerance, revealed a phenomenon of everbearing. Most changes in morphological traits were of no use. Some of them deteriorated fruit quality, first of all because of their smaller size. Alterations of that type were particularly typical of the plants, which were regenerated from calli, cultivated on media supplemented with 2,4-D. Prolonged cultivation on these media led to obtaining numerous small-fruited plants quantities of which ranged from 28% (from 'Urozhainaya CGL'-derived calli) to 100% (derived from 'Feierverk'). Moreover, a few plants produced sterile flowers, whereas quantities of small-fruited plants regenerated from calli cultivated on related media supplemented with IBA, ranged from 15% (derived from 'Urozhainaya CGL') to 35% (derived from 'Lvovskaya Rannyaya').

CONCLUSIONS

Tolerance to phytotoxins from *P. cactorum* almost in all cases indicated resistance/tolerance to the pathogen itself. The majority of plants regenerated from calli that kept intensive growing and proliferating on selective media, possessed resistance/tolerance to the fungus.

Tolerance to phytotoxins of *B. cinerea* was not equal to resistance/tolerance to the fungal disease, but tissue screening on media containing toxins from the fungus may be a selection tool to obtain genotypes with the desirable trait, since its employment resulted in several tolerant lines.

Testing for resistance to the diseases using leaf inoculation followed by incubation of infected leaves in Petri dishes proved to be reliable enough to consider this technique as an alternative to crown inoculation. This method is faster and inexpensive, and may be successfully used at least at early stages of selecting work, with a subsequent evaluation of the most promising lines in field conditions, using more reliable but cost-consuming methods.

Plants, regenerated from most callus tissue specimens selected for tolerance to salinity on selective media, were significantly more tolerant to the stressor compared with initial cultivars, which was confirmed by testing *in vitro*, followed by laser analyses of leaf tissue microstructure and, finally, by the method of leaf diagnostics. The three methods proved being highly reliable to obtain needed estimates at different phases of selecting work.

Tissue selection allowed obtaining lines, resistant/tolerant to *B. cinerea*, *P. cactorum* and excessive salinity, possessing important traits of initial cultivars. Optimal media compositions were found for micropropagation of nearly all selected lines depending on their origin and plant characteristics.

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