

## Evaluation of strawberry (*Fragaria x ananassa* Duch.) cultivar's salt stress tolerance on the basis of S<sub>1</sub> seeds *in vitro* germination

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### Abstract

Ten strawberry cultivars and one breeding clone were evaluated for their salt tolerance under *in vitro* culture conditions on the basis of S<sub>1</sub> seed germination. Salinity stress was simulated by the mixture of four salts (75 mM NaCl, 50 mM Na<sub>2</sub>SO<sub>4</sub>, 10 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>) to balance different types of ions. Germination of seeds was observed for 10 weeks. Coefficients describing reduction of seed germination (RSG and MR) were calculated. Cluster analysis separated all clones into 3 groups. The first cluster comprises cultivars 'Toringa', 'Pegasus', 'Ottawa' and clone Pau/26 with low germination under *in vitro* culture conditions. The last selection seems not to be affected by applied salt concentration. The second group was formed by moderately (60-69%) germinating cultivars: 'Silver Jubilee', 'Fraginette', 'Tenira', 'Governor Simcoe' and 'Lvovskaya Rannaya'. Out of them 'Governor Simcoe' showed low reduction of seed germination (RGS 33%). The third group was represented by well-germinating cvs. 'Fletcher' and 'Senga Litessa'. 'Fletcher' was tolerant to applied salt concentration (RSG 17.4%). Obtained data suggest that cluster analysis reflected mainly tested cultivars' general ability to germinate at *in vitro* conditions. On the basis of the mean reduction in 10<sup>th</sup> week values (MR<sub>10</sub>) genotypes can be arranged from most susceptible to most salt tolerant in the following order: 'Toringa' > 'Ottawa' > 'Pegasus' > 'Fraginette' > 'Lvovskaya Rannaya' > 'Silver Jubilee' > 'Tenira' > 'Governor Simcoe' > 'Senga Litessa' > 'Fletcher' > Pau/26. Clone Pau/26 showing the lowest MR<sub>10</sub> value in the test was selected at *in vitro* conditions as an individual plant able to germinate and grow on medium containing 200 mM NaCl. This implies that at least in the case of this genotype ability to germinate and sustain growth on salt containing medium was transmitted to S<sub>1</sub> generation.

**Key words:** *Fragaria x ananassa*, *in vitro* selection, salinity tolerance, strawberry breeding.

### Introduction

Soil salinity is an important factor limiting agricultural land productivity in many regions of the world<sup>21, 23</sup>. Inappropriate agricultural practices and predicted effects of global climate change will further deepen this problem<sup>50</sup>. On the other hand in other places only salty water is available for crop irrigation. The problem of soil and water salinization can be, however, partially overcome by breeding and selection of new varieties. According to Priel<sup>34</sup> water containing up to 2500 mg of chlorides per litre can be successfully used for irrigation salt resistant cultivars of melon, capsicum, kohlrabi, tomato, lettuce, potato, almond, pear and grape at desert regions. Differences in salt tolerance between varieties (and wild closely related species that can be used as a source of resistance genes) available to breeders have been reported in numerous plants e.g. alfalfa<sup>20</sup>, chickpea<sup>27</sup>, citrus<sup>39</sup>, cotton<sup>2</sup>, eucalyptus<sup>32</sup>, pepper<sup>12</sup>, potato<sup>42</sup>, rice<sup>40</sup>, rose<sup>46</sup> and tomato<sup>10, 16, 17, 25, 29-31</sup>.

Strawberry (*Fragaria ananassa* Duch.) is one of the crops showing low tolerance to salinity. According to Hancock et al.<sup>18</sup>, commercial strawberry cultivars differ in their sensitivity to salt and sensitive, semi-tolerant and tolerant clones can be identified in existing stock of cultivars. Additional source of resistance to salt can provide naturally selected ecotypes that grow along the coast of America<sup>19</sup>.

Choice of the best strategy for environmental stress tolerance improvement is a subject of plant breeder's discussions<sup>11</sup>. Final

decision has to be based on thorough analyzes of multiple biological and technical aspects of breeding process. Tal<sup>45</sup> has pointed out conditions indispensable for salt tolerance improvement: a) presence of genetic variability in stress reaction of varieties and/or wild relatives, b) availability of the screening methods effective in big populations and c) establishment of stringent selection criteria.

To increase variability in reaction to salt stress *in vitro* techniques can be used. Somaclonal variation<sup>24</sup> was exploited for salt tolerance improvement<sup>7, 8, 13, 22, 26, 34, 37, 41, 44, 47</sup>. Advantages of somaclonal variation rely on a big number of mutation events that can be generated in small space of *in vitro* lab and can be screened in strictly controlled conditions. This technique has, however, some disadvantages, like difficulties in plant regeneration from selected cell lines or the high level of abnormal regenerants.

Genetic engineering appears to be another very potent tool in quest for salinity resistance (reviewed by Rathinasabapathi<sup>36</sup> and Borsani et al.<sup>9</sup>). Promising results have been reported for tobacco<sup>1, 43</sup>, alfalfa<sup>48</sup> and tomato<sup>51</sup>. This type of approach directly targets the problem but is relatively complicated and expensive. Another factor that cannot be overlooked, especially in Europe, is growing number of consumers rejecting genetically modified food products.

In this paper we evaluate salt tolerance of segregating S<sub>1</sub> seed

populations derived from 10 strawberry cultivars and one breeding clone according to modified procedure developed by Esensee et al.<sup>15</sup> and Wright and Hughes<sup>49</sup>. The method of *in vitro* selection for salt tolerance is also described and resultant clone Pau/26 was used to verify the effectiveness of the proposed technique.

### Materials and Methods

In the experiment seeds obtained after self pollination of ten randomly chosen strawberry (*Fragaria x ananassa* Duch.) varieties ('Fletcher', 'Fraginette', 'Governor Simcoe', 'Lvovskaya Rannaya', 'Ottawa', 'Pegasus', 'Senga Litessa', 'Silver Jubilee', 'Tenira', 'Toringa') and one breeding clone Pau/26 were used. Clone Pau/26 was selected in the following procedure. Seeds from open pollination were collected from cv. Paula plants grown in the Department of Genetics and Horticultural Plants Breeding, Agricultural University, Lublin field strawberry germplasm collection comprising over 70 cultivars and breeding clones. In the obtained seeds high genetic diversity was expected in result of: a) random chromosomes segregation and crossing-over during megasporogenesis in mother plants, b) random pollination by the pollen from over 70 cultivars and clones of different origin growing in the proximity of the maternal plants.

Tested  $S_1$  populations might be affected by inbreeding, as strawberry is known to be prone to inbred depression. Developed testing procedure was designed in the way that allowed minimising the influence of inbred depression on final assumptions.

***In vitro* selection procedure:** Seeds were surface sterilized in sodium hypochloride for 2 h, rinsed three times in sterile distilled water (for 5, 10 and 15 min., placed on PT0 medium in Petri dishes (10 cm of diameter). PT0 medium is composed of macro- and microelements according to Murashige and Skoog<sup>33</sup>, 100 mg dm<sup>-3</sup> of m-inositol, 20 g dm<sup>-3</sup> of sucrose, 7 g dm<sup>-3</sup> of agar, pH 5.65 ± 0.02 adjusted with 0.1 N NaOH and 0.1 N HCl prior to autoclaving, sterilisation for 20 min. in 121°C. Dishes with seeds were placed in 5°C in the dark for the period of 8 weeks for vernalization. Afterwards seeds were transferred on the freshly prepared PT0 medium supplemented with 200 mM of NaCl and kept at constant temperature of 23°C, 16/8h day/night photoperiod. After 10 weeks 35 of best performing seedlings out of 5000 seeds were selected and moved on PT0 medium. From seedlings that the fastest recovered after stress removal 14 individual clones were developed including Pau/26.

***In vitro* testing procedure:**  $S_1$  seeds from 10 varieties and one breeding clone were subjected to the sterilisation and vernalization procedure identical to that described above. Afterwards seeds were transferred on the control (PT0) and test medium (PT0 supplemented with 75 mM NaCl, 50 mM Na<sub>2</sub>SO<sub>4</sub>, 10 mM CaCl<sub>2</sub> and 5 mM MgCl<sub>2</sub>). The pH adjustments and sterilisation were as described previously. On the control medium (salt-free) 400 seeds were tested (4 Petri dishes-replications, 100 seeds-objects per dish). For saline medium 600-1000 seeds (6-10 dishes with 100 seeds on each) depending on the cultivar were analysed. Culture conditions (temperature, light) were as for selection procedure. Germination was observed in one-week intervals for the period of 10 weeks. Seedlings with two fully developed cotyledons were scored as 'germinated'. The influence of salt on germination was calculated in two ways:

1. Mean reduction (MR) of seed germination caused by salt calculated as difference between mean germination on control ( $\bar{n}_0$ ) and mean germination on salt containing media ( $\bar{n}_s$ ) according to formula:  $MR = \frac{\bar{n}_0 - \bar{n}_s}{\bar{n}_0} \cdot 100 [\%]$

2. Reduction of seed germination (RSG) was calculated with following formula:

$$RSG = \left( 1 - \frac{N_s}{N_0} \right) \cdot 100 [\%]$$

where  $N_s$  and  $N_0$  are numbers of the seeds germinating on salt-containing media and the number of respective seeds on control. Mean numbers of seeds germinating on control and stress media during 10 weeks were standardized to obtain Pearson-moment correlation coefficient and Euclidean distance matrices. Canonical and cluster analyses were performed using respective matrices with NTSYSpC 2.10q package<sup>38</sup>.

### Results

Cultivars' reaction to salt stress applied *in vitro* during 10-week long period (with SD values) is shown in the Figs 1a-k and MR values are in Table 1. On the basis of MR in 10<sup>th</sup> week (MR<sub>10</sub>) genotypes can be arranged from most susceptible to most salt tolerant in the following order: 'Toringa' > 'Ottawa' > 'Pegasus' > 'Fraginette' > 'Lvovskaya Rannaya' > 'Silver Jubilee' > 'Tenira' > 'Governor Simcoe' > 'Senga Litessa' > 'Fletcher' > Pau/26. Cluster analysis was applied to find groups of cultivars with similar characteristics of seed germination on control and stress media. On the UPGMA dendrogram (Fig. 2) all clones were separated into 3 groups. The first cluster comprises cultivars 'Toringa', 'Pegasus' and 'Ottawa' with low germination under *in vitro* culture conditions. The lowest germination on control was observed for clone Pau/26 (20.6%). This selection, however, seems not to be affected by applied salt concentration. High reduction of seed germination in stress conditions at the beginning of experiment (98%) was followed by decline during subsequent weeks of the *in vitro* test (Table 2). The second cluster was formed by moderately (60-69%) germinating cultivars: 'Silver Jubilee', 'Fraginette', 'Tenira', 'Governor Simcoe' and 'Lvovskaya Rannaya'. Out of them 'Governor Simcoe' showed low RGS (33%). The third group was represented by well-germinating cvs. 'Fletcher' and 'Senga Litessa'. 'Fletcher' was tolerant to applied salt concentration (RSG 17.4%). Obtained data suggest that cluster analysis reflects mainly tested cultivars' general ability to germinate at *in vitro* conditions.

The influence of all parameters scored on total variability of material was established (Table 3). Scores at control and salt media strongly (range 0.76-0.97) influenced the value of the first principal component (PC) which explained 78% of differences observed. The only exception was seed germination on salt after the first week of experiment. This suggests that the first PC explains variation in germination ability and was determined by genotypic and phenotypic properties of seeds. The values influencing the second PC were low both for control (range -0.59 to 0.31) and salt (range -0.14 to 0.48). Interestingly these values were generally negative at control and positive for salt. This suggests that the second PC reflected major reaction to salt. The 3<sup>rd</sup> principal component had the lowest (3.8) share in total variation observed and was explained mainly by germination in the first week on salt-containing medium.

Two-dimensional plot reflecting distribution of cultivars along

**Table 1.** MR values in percent during ten consecutive weeks of observations.

week	Fletcher	Fraginette	Governor Simcoe	Lvovskaya Rannaya	Ottawa	Pau26	Pegasus	Senga Litessa	Silver Jubilee	Tenira	Toringa
1	69.3	23.0	12.5	10.0	0.7	11.8	7.2	48.3	27.0	15.4	7.0
2	23.5	32.6	10.2	19.7	19.5	7.3	2.6	31.0	29.0	28.4	11.8
3	14.1	38.9	19.5	31.1	45.1	4.0	10.1	35.6	44.9	41.8	14.6
4	9.0	40.4	19.4	55.7	35.1	2.9	14.8	35.0	44.7	37.4	19.5
5	5.6	42.8	20.2	55.3	34.6	1.1	20.7	28.8	61.8	37.5	25.4
6	4.8	40.0	14.3	53.7	38.2	1.6	31.5	16.8	58.1	34.4	36.1
7	4.3	39.7	15.1	46.2	40.9	1.8	32.2	16.6	56.7	31.7	39.0
8	3.4	39.8	14.3	39.6	41.1	0.9	30.4	16.2	41.0	29.8	48.1
9	3.5	38.3	15.0	34.1	42.5	1.1	37.9	12.7	39.8	26.1	51.0
10	3.1	37.9	15.1	32.8	45.1	1.0	41.3	11.5	32.3	24.1	52.1

**Table 2.** Coefficients and eigenvalues for the first three principal components based on Pearson-correlation matrix between genotypes.

Week	PC1		PC2		PC3	
	Control	Salt	Control	Salt	Control	Salt
1	0.867	0.567	0.314	-0.139	0.168	0.801
2	0.938	0.834	0.199	0.484	-0.028	0.109
3	0.956	0.861	-0.042	0.485	-0.213	0.008
4	0.947	0.857	-0.224	0.471	-0.059	-0.073
5	0.915	0.885	-0.354	0.435	-0.114	-0.085
6	0.876	0.935	-0.476	0.326	-0.012	-0.067
7	0.864	0.948	-0.502	0.251	-0.040	-0.038
8	0.834	0.972	-0.540	0.143	-0.028	-0.017
9	0.800	0.972	-0.572	0.085	-0.013	0.014
10	0.764	0.970	-0.595	0.037	-0.002	0.012
Eigenvalues	15.59		2.86		0.77	
Percent of total variance	77.94		14.32		3.83	

the first and second PC resembles groupings obtained by cluster analysis. As the first PC reflects most of variation not corresponding with reaction to salt we ignored it and resolved clones along second and third PC (Fig. 3). The highest toleration to salt (based on RSG mean values) was observed for 'Fletcher' and Pau/26. However, 'Governor Simcoe' and 'Senga Litessa' also showed small vulnerability to salt (RSG 33%). The growth of most strawberries was hampered on salt at the rate ranging from 53% for 'Tenira' to 77% for 'Toringa'. Higher sensitivity was found in 'Fraginette', 'Lvovskaya Rannaya' and 'Silver Jubilee'.

### Discussion

Strawberry (*Fragaria x ananassa* Duch.) is very sensitive to salinity<sup>14</sup>. First symptoms appear after soil extract electric conductivity rises above 2 dS·m<sup>-1</sup><sup>28</sup>. Salt stress symptoms are visible at first on the older leaves. They have a form of tip burnings that grow towards the leaflet base. Younger leaves are less vulnerable. They curl but remain alive. NaCl reduces leaf number and area, shoot dry weight, number of crowns and inflorescences<sup>3</sup>, as well as leaf water, osmotic and turgor potentials<sup>5</sup>. Excessive salt influences also fresh and dry weight of the fruits<sup>4,6</sup> thus directly decreasing the yield and economic effectiveness of strawberry production. These observations are stimuli for improvement of salt tolerance in newly developed varieties. Resistance to salt stress can be improved by introduction of genes from wild relatives, for instance *F. chiloensis*<sup>15,49</sup>. However, prior to wide crosses we should assess the variation for this trait in cultivated strawberries and verify the effectiveness of accumulation of genes that are present in this genetic pool. Observations of 10 cultivars and one advanced breeding clone

have shown evident differences between them in sensitivity to salt stress during *in vitro* germination. These differences have in our opinion genetic background and might be exploited in breeding. Most of variation observed was due to general ability of seeds to germinate. This pushed us to search of the best way to compare differences in salt tolerance between varieties.

Indexes MR and RSG have been calculated. RGS values reflect general reaction of tested seed populations to salinity and do allow comparisons between populations. MR values at the 10<sup>th</sup> week have more practical application. If vernalized seeds are placed on medium in Petri dishes, almost all viable seeds will germinate until this time point (see Fig. 1a-k) and (if salt concentration was chosen properly) differences in appearance between less and more

tolerant seedlings will be evident. The test of this type cannot be carried out any longer as the medium dries out to the level inhibiting further germination and seedling growth. In our opinion 10<sup>th</sup> week is the right time to select the best looking seedlings for further evaluation.

Varieties differ in terms of the reaction time and seed's adaptation ability to stress conditions. All clones tested but 'Ottawa' and 'Lvovskaya Rannaya' showed delay and reduction of seed germination in the first week in the range of 87-100%. In case of salt tolerant cultivars 'Fletcher' and clone Pau/26, mechanism of adaptation is efficient and after 4 weeks RSG declined below 10%. Cultivars 'Governor Simcoe' and 'Senga Litessa' were able to reduce influence of salt below 20% after 5<sup>th</sup> week.

Experiment revealed differences in reaction to salt at germination and early growth stages between S<sub>1</sub> seeds collected from investigated strawberry clones. The reaction to salt stress seems to be independent from ability to germinate *in vitro*, and tolerant genotypes could be identified in both groups (with high and low *in vitro* seed germination rate). All cultivars can be divided into 3 groups on the basis of tolerance reaction mode. Germination of seeds in the first group is strongly affected at the beginning of observation. Reaction in the second group concentrates on 4<sup>th</sup> and 5<sup>th</sup> week of culture, while for other cultivars reduction of germination is growing along the time of experiment. This suggests that for evaluation of large seed samples (e.g. variety comparison) tests shorter than 10 weeks can be performed.

We can conclude that improvement of strawberry's tolerance to salinity through conventional breeding is possible. Both, tolerant and susceptible clones have been identified and can be used for generation of segregating population for mapping and

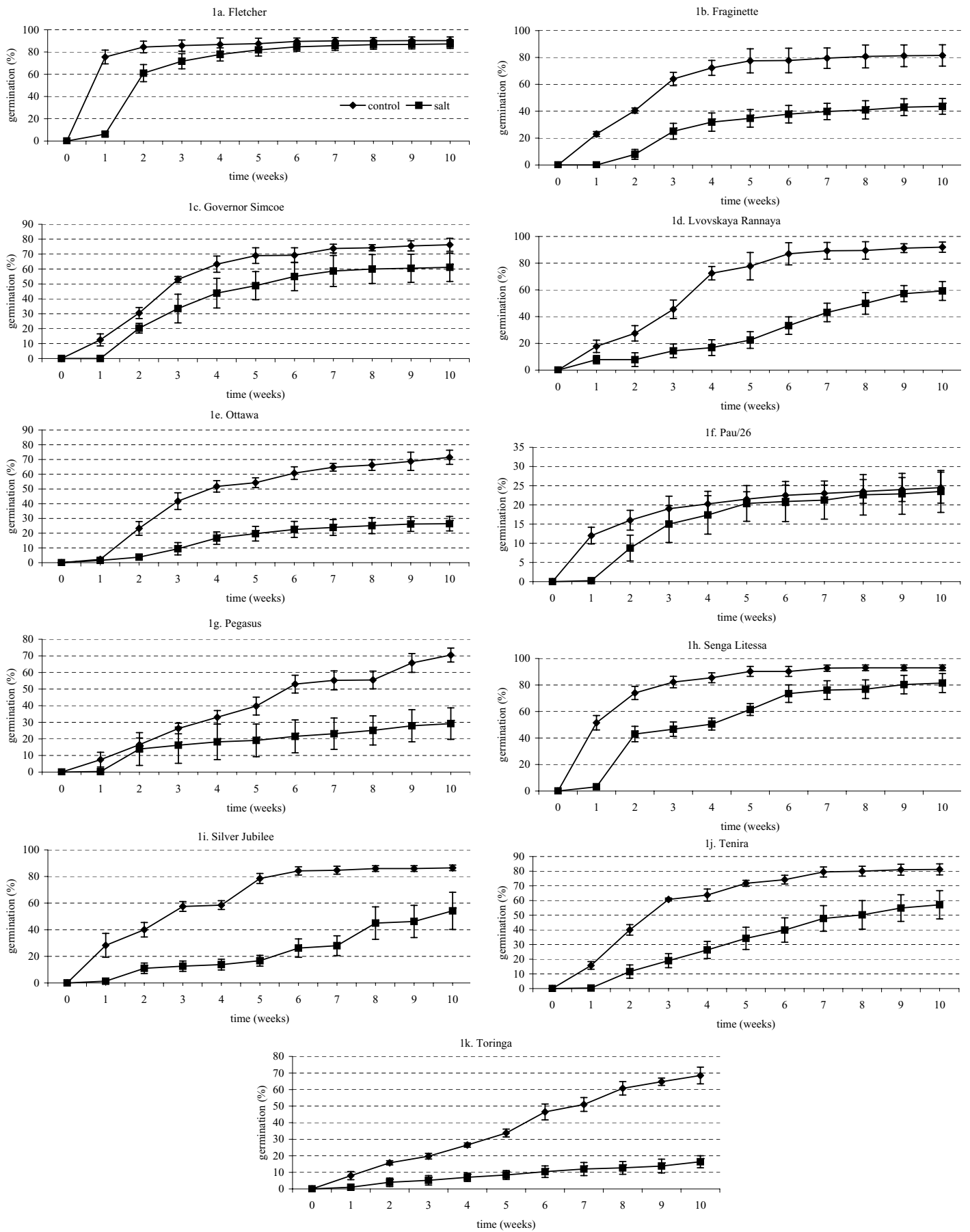
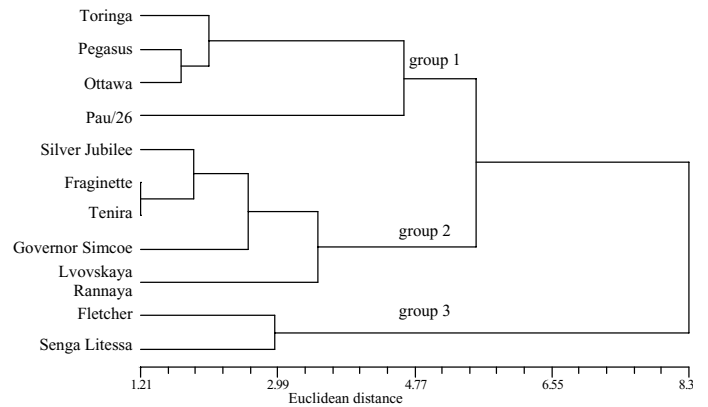


Figure 1a-k. Germination of 10 strawberry varieties and clone Pau/26 on salt and control media, the bars are showing SD values.

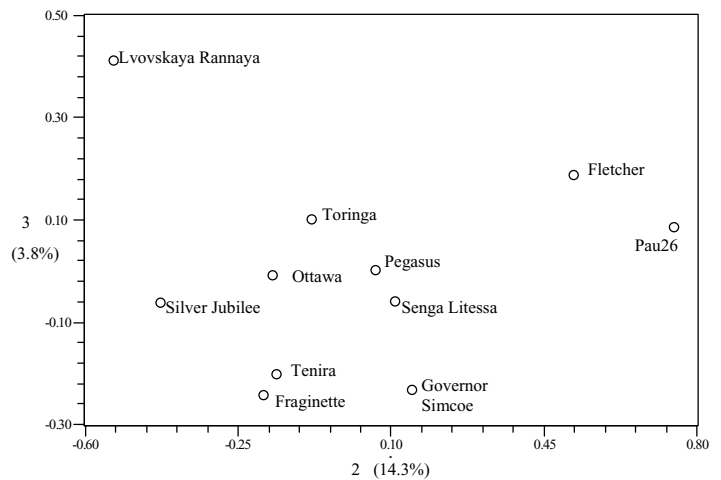
**Table 3.** Seed germination (%) on control and its reduction (RSG) in saline conditions during ten consecutive weeks of observations.

week	Fletcher		Fraginette		Governor Simcoe		Lvovskaya Rannaya		Ottawa		Pau/26		Pegasus		Senga Litessa		Silver Jubilee		Tenira		Toringa		
	control	RSG	control	RSG	control	RSG	control	RSG	control	RSG	control	RSG	control	RSG	control	RSG	control	RSG	control	RSG	control	RSG	
1	75.5	91.8	23.0	100.0	100.0	12.5	100.0	17.8	56.1	2.3	30.9	12.0	97.9	7.5	96.0	51.5	93.9	28.3	95.4	15.8	97.9	8.0	87.5
2	84.5	27.8	40.5	80.5	30.5	33.3	27.5	71.6	23.3	83.8	16.0	45.3	16.5	15.8	74.0	41.9	41.9	40.0	72.5	40.0	71.1	15.8	74.6
3	85.8	16.4	64.0	60.8	53.0	36.8	45.5	68.4	41.8	77.4	19.0	21.1	26.3	38.3	82.3	43.3	43.3	57.5	78.1	60.8	68.7	19.8	73.7
4	86.8	10.3	72.3	55.8	63.3	30.7	72.5	76.8	51.8	67.8	20.3	14.2	33.0	44.8	85.5	40.9	40.9	58.5	76.4	63.8	58.7	26.5	73.6
5	87.5	6.4	77.5	55.2	69.0	29.2	77.8	71.1	54.3	63.7	21.5	5.2	39.8	51.9	90.3	31.9	31.9	78.5	78.7	71.8	52.3	33.8	75.1
6	89.5	5.4	77.8	51.4	69.3	20.6	87.0	61.7	60.8	62.9	22.5	7.2	53.0	59.4	90.3	18.6	18.6	84.3	68.9	74.3	46.3	46.5	77.6
7	90.0	4.8	79.5	49.9	73.8	20.5	89.3	51.7	64.8	63.1	23.0	7.6	55.3	58.2	92.8	17.9	17.9	84.7	67.0	79.5	39.9	51.0	76.5
8	90.0	3.8	80.8	49.2	74.3	19.2	89.5	44.2	66.3	62.1	23.5	3.7	55.5	54.8	93.0	17.4	17.4	86.0	47.7	80.0	37.2	60.8	79.1
9	90.3	3.8	81.3	47.1	75.5	19.9	91.3	37.3	68.8	61.9	24.0	4.7	65.8	57.6	93.0	13.6	13.6	86.0	46.3	81.0	32.2	64.8	78.7
10	90.3	3.4	81.5	46.5	76.3	19.8	92.0	35.7	71.5	63.0	24.5	4.1	70.5	58.6	93.0	12.4	12.4	86.5	37.3	81.3	29.7	68.5	76.1
mean	87.0	17.4	67.8	59.6	59.8	33.0	69.0	57.5	50.6	63.6	20.6	21.1	42.3	53.5	84.6	33.2	33.2	69.0	66.8	64.8	53.4	39.6	77.2
variation*	5.2	156.6	29.7	29.1	36.4	74.0	40.9	25.9	44.1	21.6	19.4	141.5	50.0	37.6	15.6	74.0	74.0	31.3	26.7	33.2	39.8	55.0	5.3

\* variation = (standard deviation/mean)\*100 [%]



**Figure 2.** UPGMA dendrogram based on values of Euclidean distance.



**Figure 3.** Two-dimensional plot representing distribution of genotypes reaction on the second and third principal components.

tracing QTLs explaining salt stress tolerance. *In vitro* culture offers both tool for selection and system of salt tolerance evaluation. This approach is consumer oriented, effective and inexpensive.

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