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Stability of *Verticillium dahliae* resistance in tissue culture-derived strawberry somaclones

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Abstract

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Somaclonal variation is a novel source of useful traits in strawberry breeding. In our previous work we selected K40 clone from *in vitro* cultures of strawberry cv. Elsanta, which was characterized by high resistance to *Verticillium dahliae*. The objective of this work was to evaluate the stability and transmission of this trait during *in vitro* shoot proliferation and traditional clonal propagation from runners. K40, Elsanta and Senga Sengana plants were propagated *in vitro* for 45 generations/passages or for 4 generation of clonal propagation from runners. The resistance/susceptibility of such derived plants to *V. dahliae* was tested in controlled conditions in a greenhouse. Irrespective of the method of propagation, the plants of K40 clone were much more resistant to *V. dahliae* than the plants of the original cultivar and the plants of 'Senga Sengana', which is a cultivar considered as resistant to Verticillium wilt in field conditions. This proves that this trait elicited at somaclonal variant is stable and transferable both during *in vitro* micropropagation and clonal plant propagation from runners.

Keywords: somaclonal variation; *in vitro* selection; plant pathogen; Verticillium wilt

Strawberry (*Fragaria* × *ananassa* Duch.) is one of the most important fruit in the temperate climatic zone. World production exceeds 4,516,810 t annually. In Europe the biggest producers of strawberries are Spain (289,900 t), Germany (155,828 t) and Poland (150,151 t) (FAOSTAT 2012). Among the most important factors limiting production are fungal diseases, especially those caused by soil-borne pathogens, which may generate heavy losses in favourable seasons. The most difficult to control is Verticillium wilt, caused by the fungus *Verticillium dahliae* Kleb. (*Hyphomycetes*, formerly *Deuteromycotina*, *Fungi imperfecti*). The pathogen infects plants through root system. Upon passing endodermis, conidia are formed and released into the xylem system where they develop hyphae, which block the flow of water and nutrients. Verticillium wilt symptoms on strawberry include wilting, chlorosis, stunting and finally necrosis (Fradin, Thomma 2006). Intensity of the disease depends on the cultivar, soil type, pathogen's soil infestation and on occurrence of symbiotic microorganisms in rhizosphera (Nallanchakravarthula et al. 2014). On heavily infested plots up to 90% of the susceptible plants may die during the first year after planting (Meszka, Bielenin 2009).

The control of the pathogen is difficult. Pre-planting soil fumigation or pesticide treatment is costly and very damaging to the environment. Alternative methods like soil solarisation, crop rotation or soil amendments with animal or plant-derived composts are only partially effective (Goicoechea 2009). The recommendations to farmers are to plant strawberries (and other susceptible crops) on

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soils with low pathogen's population, but this is difficult to achieve because *Verticillium dahliae* has very wide host range and its infective microsclerotia can survive dormant in the soil for up to 15 years (Wilhelm 1955; López-Escudero, Mercado-Blanco 2011). Thus, one of the major priorities in strawberry breeding programmes is breeding for resistance to *Verticillium* spp. (Żebrowska 2011). However, complicated genetics and narrow germplasm base of this hybrid octoploid species makes classical breeding difficult (Amil-Ruiz et al. 2011). Some progress can be expected due to the recent construction of strawberry genome-wide SSR linkage map (van Dijk et al. 2014), but reliable Quantitative Trait Loci (QTL) conferring Verticillium wilt resistance/tolerance is still lacking. The attractive alternative is selection of tissue culture-induced somaclonal variants, which has been shown to be a source of useful traits for improvement of many crop plants (Carp 1995).

A method for inducing and *in vitro* selecting somaclonal variants of strawberry with enhanced resistance to *Verticillium dahliae* has been developed (Sowik et al. 2001). The clone K40, selected from tissue culture of strawberry cv. Elsanta was characterized by much higher resistance to *Verticillium dahliae*, both *in vitro* and *ex vitro*, than the original cultivar and comparable to resistance of cv. Senga Sengana, which is considered as resistant to Verticillium wilt. Besides enhanced resistance to *Verticillium dahliae*, the morphology of the somaclone was also affected; its leaf petioles were longer, leaf blades larger, crown diameter bigger and root system better developed than in the plants of the original cultivar (Sowik et al. 2003).

Somaclonal variation may have genetic (mutations, gene duplication, polyploidy) or epigenetic background (DNA methylation, transposable element movement, histone acetylation) (Peschke, Phillips 1992; Kaeppler et al. 2000; Miguel, Marum 2011). In the latter case, the traits elicited as somaclonal variants may revert to the original phenotype in a few clonal or sexual generations (Jain 2001; Vazques 2001). Therefore, the stability of somaclonal traits has to be tested during several generations. The objective of the present research was to evaluate stability and transferability of resistance to *Verticillium dahliae* in K40 somaclone during long-term *in vitro* micropropagation and traditional propagation from runners in the field.

MATERIAL AND METHODS

Plant material. The experiments were performed on 3 genotypes of strawberry (*Fragaria* × *ananassa* Duch.):

– K40 somaclone resistant to *Verticillium dahliae*, selected *in vitro* from cv. Elsanta,
– cv. Elsanta (Els.) highly susceptible to Verticillium wilt,
– cv. Senga Sengana (Sen.) resistant to Verticillium wilt.

In order to evaluate stability and transferability of the resistance during long-term micropropagation and traditional propagation from runners, plants derived from shoots multiplied *in vitro* for 45 generation/passages (later on referred to as "old": "K40 O", "Els. O" and "Sen. O" for K40 clone, cvs Elsanta and Senga Sengana, respectively) were compared with plants derived from "new" *in vitro* cultures established from plants of the same genotypes propagated traditionally in the field during four seasons (4 generations) and then micropropagated during 8 passages (later on referred to as "K40 N", "Els. N" and "Sen. N", respectively).

The *in vitro* strawberry shoot cultures were established from runner tip meristems taken from virus-free mother plants, grown in a greenhouse. A basal medium of Boxus (1974) was applied, which contained macroelements according to Knop (1965), microelements and vitamins according to Murashige and Skoog (1962) and supplemented with glycine (2 mg/l), inositol (100 mg/l), glucose (40 g/l), IBA (1 mg/l), BA (0.1 mg/l), GA_3 (0.1 mg/l), and solidified with Difco bacto agar (8 g/l). pH of the medium was adjusted to 5.6–5.7 prior to autoclaving. The medium for shoot proliferation contained higher concentrations of IBA (0.5 mg/l) and BA (0.5 mg/l). The *in vitro* cultures were maintained in a growth chamber at 23°C/18°C (day/night), under white light (Philips fluorescent tubes 'TL'D 36W; Philips Lighting, Netherlands) with the quantum irradiancy 55 μmol/m^2·s and 16 h photoperiod. Every six weeks the shoots were transplanted on fresh medium.

Shoot rooting and plant acclimation. Rooting and acclimation of micropropagated shoots was done *ex vitro*. The shoots, 15–20 mm in length, were planted in mineral wool substrate (Grodan®) and placed in a growth chamber at temperature 21–23°C and 16 h photoperiod at photon flux density (PFD) 75 mmol/m^2·s (Philips fluorescent tubes 'TL'D 36W). For the first week the plants were

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watered once a day with 250 mg/l soluble fertilizer (Professional Peters®, Scotts Company, Everris International B.V., Geldermalsen, Netherlands) (10% N, 52% P, 10% K), and for subsequent three weeks watered every second day with a solution of 800 mg/l Peters® Professional fertilizer consisting of 20% N, 10% P, 20% K, alternating with water. After 7 weeks the rooted shoots were transplanted to pots filled with mixture (1:5, v/v) of sand and plant growth substrate "Blumenerde" (Athena, Germany) and grown in the greenhouse. During the growth the plants were watered once a day.

Analysis of morphology of micropropagated shoots. Shoots kept in *in vitro* cultures for 45 passages ("old"), and for 8 passages following runners propagation, ("new") cultures, were grown for 6 weeks on propagation medium in the growth chamber in conditions as above, except light intensity, which was 55 mmol/m^2·s. The following morphological traits were evaluated:

– axillary shoot propagation rate,
– fresh mass of shoots and leaves,
– dry matter content in shoots and leaves,
– leaf number and area,
– length of petioles.

The experiment was completely randomised with 10 replications, where a replication consisted of 2 jars, each containing initially 12 shoots of each cultivar.

Analysis of morphology of acclimated plants. After 10 weeks of growth in the greenhouse the following morphological traits were evaluated:

– mean leaf area,
– mean petiole length,
– mean root length,
– crown diameter,
– fresh and dry (drying for 48 h at 80°C) mass of leaves and roots,
– fresh mass of crown and runners,
– number of runners and daughter plants developed.

The experimental design was completely randomised with 3 replications, where a replication consisted of 8 potted plants of each genotype.

Evaluation of susceptibility to *Verticillium dahliae* infection. After 10 weeks of growth in the greenhouse, plants' susceptibility to *Verticillium dahliae* was tested in the greenhouse conditions as follows. 26 isolates of the pathogen were obtained from the heavily infested experimental field and used in a mix for testing strawberry plants susceptibility to Verticillium wilt. The isolated fungi were cultured for 14 days on Malt Extract Agar medium (Sigma-Aldrich, Saint Louis, USA) and then transferred to liquid malt extract medium. After 21 days the fungal mycelium was homogenized together with the growth medium and inoculated on sterile substrate containing sand, cornmeal and water (10:1:2, v/v/v). After 3 weeks of incubation at 21°C, when the whole substrate was overgrown with the fungus, it was used for inoculating soil substrate in the pots with tested plants; the upper 3 cm of the healthy soil substrate in the pots were removed and replaced with the substrate with the pathogen. In order to facilitate faster infection, the plant's roots were slightly injured with sharp stick. The control plants were grown in soil substrate treated with sterile mixture of sand and cornmeal. During the entire experiment the plants were regularly watered using drip irrigation applied to each pot. Susceptibility to the infection by the pathogen was estimated weekly by recording symptoms of disease (infection rate) according to the following scale:

0 – healthy shoots, no symptoms on leaves,
1 – shoots with single leaves showing infection symptoms (wilting, yellowish-brown appearance),
2 – up to 25% of leaves showing infection symptoms,
3 – up to 50% of leaves showing infection symptoms,
4 – up to 75% of leaves showing infection symptoms,
5 – 100% of leaves showing infection symptoms.

On the basis of these observations, the disease index (*DI*) was calculated according to McKinney (1923):

$$DI = (\Sigma vn)/(NV) \times 100$$

where:

v – numeric value corresponding to the class
n – number of leaves or fruits assigned to the class
N – total number of the leaves or fruits in the replication
V – numeric value of the highest class

The experimental design was completely randomised with 3 replications, where a replication consisted of 8 potted plants of each genotype.

The results of all experiments were elaborated statistically by two-factorial analysis of variance using Statistica 5.5 software and the significance of

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Table 1. Morphology of *in vitro* strawberry shoots micropropagated *in vitro* during 45 passages (O) and those propagated traditionally for 4 generations and then micropropagated for 8 passages (N)

Morphological traits	Genotypes/propagation					
	K40 N	K40 O	Els. N	Els. O	Sen. N	Sen. O
Micropropagation rate	2.4 ± 0.56^{b}	2.1 ± 0.32^{ab}	2.1 ± 0.39^{ab}	1.9 ± 0.30^{a}	2.5 ± 0.50^{b}	3.2 ± 0.64^{c}
Shoot fresh mass (mg)	185 ± 20.0^{ab}	196 ± 31.1^{b}	184 ± 36.7^{ab}	165 ± 21.7^{a}	166 ± 19.2^{a}	166 ± 21.1^{a}
Leaf fresh mass (mg)	54.1 ± 1.64^{a}	66.2 ± 7.73^{b}	54.4 ± 3.08^{a}	55.7 ± 8.22^{a}	56.9 ± 7.25^{a}	58.5 ± 7.32^{a}
Leaf dry matter content (%)	33.6 ± 1.14^{a}	34.0 ± 1.29^{a}	32.9 ± 0.87^{a}	33.8 ± 0.85^{a}	32.7 ± 2.09^{a}	32.9 ± 1.15^{a}
Length of shoots (mm)	13.7 ± 1.12^{c}	11.8 ± 1.25^{b}	12.3 ± 1.08^{b}	10.9 ± 0.91^{a}	17.3 ± 1.56^{e}	15.3 ± 0. 59^{d}
Number of leaves (pcs)	10.1 ± 3.07^{cd}	9.4 ± 3.11b^{c}	8.9 ± 2.17^{ab}	8.1 ± 2.57^{a}	10.7 ± 2.61^{d}	10.9 ± 2.72^{d}
Leaf area (mm^2)	60.1 ± 4.17^{a}	66.9 ± 8.17^{c}	61.3 ± 5.34^{a}	65.4 ± 3.74^{b}	58.1 ± 5.59^{a}	57.3 ± 3.36^{a}

means (± SD) in the rows followed by the same letter are not significantly different according to the Duncan's *t*-test at $P = 0.05$; K40 – somaclone, Els. – cv. Elsanta, Sen. – cv. Senga Sengana

differences between means was evaluated with the Duncan's *t*-test at $P = 0.05$.

RESULTS AND DISCUSSION

The method of propagation did not have a big effect on morphology of the strawberry genotypes studied. Only small differences were observed in morphology between shoots derived from *in vitro* cultures propagated for 45 passages and those propagated by runners and then *in vitro* for 8 passages. In general, the shoots from "new" cultures were by 14–16% longer that those from the "old" ones in all genotypes, but the opposite relationship was observed in case of leaf biomass of K40 clone, micropropagation rate of cv. Senga Sengana and leaf area in K40 clone and cv. Elsanta (Table 1).

Similarly as in *in vitro* cultures, there were few differences in morphology of 10-weeks old acclimated plants of the three genotypes between those derived from cultures propagated for 45 passages and these propagated traditionally for 4 generations and then micropropagated for 8 passages. The leaf petioles of all the genotypes tested were longer in plants derived from "new" than in plants obtained from

Table 2. Morphology of strawberry plants studied after 10 weeks of growth in the greenhouse

Morphological traits	Genotypes/propagation					
	K40 N	K40 O	Els. N	Els. O	Sen. N	Sen. O
Crown diameter (mm)	12.4 ± 0.27^{c}	12.3 ± 0.59^{c}	12.1 ± 0.39^{c}	12.3 ± 0.51^{c}	9.1 ± 0.51^{a}	11.3 ± 0.27^{b}
Mean leaf area (cm^2)	55.6 ± 3.50^{d}	58.3 ± 5.88^{d}	45.2 ± 1.95^{bc}	48.5 ± 1.79^{c}	42.4 ± 0.61^{ab}	38.4 ± 1.76^{a}
Mean length of the petiole (cm)	11.0 ± 0.08^{e}	9.8 ± 0.06^{d}	8.6 ± 0.02^{b}	8.1 ± 0.07^{a}	11.1 ± 0.22^{e}	8.8 ± 0.05^{c}
Fresh mass of aboveground parts (g)	8.6 ± 0.23^{ab}	9.4 ± 1.03^{b}	7.7 ± 0.26^{a}	8.6 ± 0.04^{ab}	7.9 ± 0.04^{a}	7.7 ± 0.15^{a}
Leaf fresh mass (g)	5.9 ± 0.52^{b}	6.8 ± 0.83^{b}	5.1 ± 0.20^{a}	6.0 ± 0.16^{b}	4.9 ± 0.25^{a}	5.0 ± 0.19^{a}
Leaf dry matter content (%)	27.6 ± 0.24^{ab}	27.4 ± 2.96^{ab}	27.7 ± 0.36^{ab}	25.9 ± 0.43^{a}	28.5 ± 1.42^{ab}	29.8 ± 0.29^{b}
Mean root length (cm)	26.9 ± 2.99^{a}	27.8 ± 2.05^{a}	26.8 ± 1.54^{a}	25.9 ± 1.65^{a}	25.4 ± 1.34^{a}	26.2 ± 0.17^{a}
Root fresh mass (g)	2.4 ± 0.45^{a}	3.2 ± 0.16^{b}	2.3 ± 0.10^{a}	3.4 ± 0.19^{b}	2.5 ± 0.55^{a}	2.5 ± 0.13^{a}
Root dry matter content (%)	14.2 ± 1.00^{a}	17.8 ± 1.77^{b}	14.2 ± 0.72^{a}	16.7 ± 0.58^{b}	13.0 ± 0.44^{a}	12.7 ± 0.74^{a}

means (± SD) in the rows followed by the same letter are not significantly different according to the Duncan's *t*-test at $P = 0.05$; K40 – somaclone; Els. – cv. Elsanta; Sen. – cv. Senga Sengana; O – shoots micropropagated *in vitro* during 45 passages; N – plants propagated traditionally for 4 generations and then micropropagated for 8 passages

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Table 3. Characteristics of propagation ability of 10-week old strawberry plants in the greenhouse conditions

Propagation indice	Genotypes/propagation					
	K40 N	K40 O	Els. N	Els. O	Sen. N	Sen. O
Number of runners produced (pcs/plant)	3.2 ± 0.52^{b}	2.4 ± 0.39^{ab}	2.5 ± 0.64^{ab}	2.7 ± 0.29^{ab}	2.1 ± 0.14^{a}	2.4 ± 0.11^{ab}
Number of daughter plants produced (pcs/plant)	3.5 ± 0.68^{b}	2.8 ± 0.44^{ab}	2.5 ± 0.63^{a}	2.9 ± 0.29^{ab}	2.0 ± 0.43^{a}	2.6 ± 0.43^{ab}
Root fresh mass of daughter plants (g)	7.7 ± 1.07^{a}	7.8 ± 1.84^{a}	5.5 ± 1.25^{a}	7.9 ± 0.22^{a}	5.8 ± 1.31^{a}	6.0 ± 1.09^{a}

means (± SD) in the rows followed by the same letter are not significantly different according to the Duncan's *t*-test at *P* = 0.05; K40 – somaclone; Els. – cv. Elsanta; Sen. – cv. Senga Sengana; O – shoots micropropagated *in vitro* during 45 passages; N – plants propagated traditionally for 4 generations and then micropropagated for 8 passages

the "old" cultures. In case of cv. Senga Sengana the crown diameter was larger in plants derived from "old" cultures and in case of cv. Elsanta there was a similar relationship for leaf fresh weight (Table 2). With exception of cv. Senga Sengana, the roots fresh mass and dry matter content of K40 and cv. Elsanta plants derived from "old" cultures were higher that these indices in plants derived from "new" cultures (Table 2).

Generally, the plants of K40 somaclone were more vigorous (in leaf area, length of petioles, and leaf fresh weight) than the plants of the original cv. Elsanta. Irrespective of the propagation method, they were taller and their leaf blades were larger.

The duration of *in vitro* culturing did not have a significant impact on the number of runners and daughter plants developed by mature plants (Table 3).

Table 4. Infection rate of Verticillium wilt in strawberry genotypes infected with *V. dahliae* in the greenhouse test

Weeks after inoculation	Genotypes/propagation					
	K40 N	K40 O	Els. N	Els. O	Sen. N	Sen. O
4	0.04 ± 0.2^{a}	0 ± 0^{a}	0.13 ± 0.34^{a}	0.08 ± 0.28^{a}	0 ± 0^{a}	0.04 ± 0.2^{a}
5	0.04 ± 0.2^{a}	0.13 ± 0.34^{a}	0.46 ± 0.66^{b}	0.54 ± 0.72^{b}	0.08 ± 0.28^{a}	0.08 ± 0.28^{a}
6	0.04 ± 0.2^{a}	0.13 ± 0.34^{a}	0.46 ± 0.66^{b}	0.54 ± 0.72^{b}	0.08 ± 0.28^{a}	0.08 ± 0.28^{a}
7	0.04 ± 0.2^{a}	0.13 ± 0.34^{a}	0.50 ± 0.72^{b}	0.67 ± 0.87^{b}	0.08 ± 0.28^{a}	0.08 ± 0.28^{a}
8	0.04 ± 0.2^{a}	0.13 ± 0.34^{a}	0.75 ± 0.68^{b}	0.71 ± 0.69^{b}	0.13 ± 0.34^{a}	0.08 ± 0.28^{a}
9	0.04 ± 0.2^{a}	0.13 ± 0.34^{a}	0.75 ± 0.68^{b}	0.75 ± 0.74^{b}	0.13 ± 0.34^{a}	0.10 ± 0.29^{a}
10	0.04 ± 0.2^{a}	0.13 ± 0.34^{a}	0.83 ± 0.68^{b}	1.00 ± 0.88^{b}	0.13 ± 0.34^{a}	0.21 ± 0.39^{a}
11	0.08 ± 0.19^{a}	0.13 ± 0.34^{a}	1.04 ± 0.81^{b}	1.21 ± 1.13^{b}	0.13 ± 0.34^{a}	0.21 ± 0.39^{a}
12	0.08 ± 0.19^{a}	0.13 ± 0.34^{a}	1.08 ± 0.83^{b}	1.29 ± 0.86^{b}	0.13 ± 0.34^{a}	0.25 ± 0.42^{a}
13	0.08 ± 0.19^{a}	0.13 ± 0.34^{a}	1.29 ± 1.00^{b}	1.54 ± 0.98^{b}	0.13 ± 0.34^{a}	0.33 ± 0.46^{a}
14	0.13 ± 0.22^{a}	0.19 ± 0.36^{a}	1.96 ± 1.20^{b}	2.38 ± 0.92^{c}	0.13 ± 0.34^{a}	0.33 ± 0.46^{a}
15	0.17 ± 0.32^{a}	0.38 ± 0.49^{a}	2.46 ± 1.28^{b}	2.75 ± 1.07^{b}	0.27 ± 0.44^{a}	0.52 ± 0.45^{a}
16	0.29 ± 0.46^{a}	0.38 ± 0.49^{ab}	2.92 ± 0.72^{c}	3.13 ± 0.54^{c}	0.42 ± 0.50^{ab}	0.67 ± 0.75^{b}
17	0.63 ± 0.71^{a}	0.92 ± 1.10^{ab}	3.38 ± 0.92^{c}	3.46 ± 0.51^{c}	0.92 ± 0.83^{ab}	1.17 ± 1.01^{b}
18	1.92 ± 0.67^{a}	1.67 ± 1.45^{a}	3.67 ± 1.01^{b}	3.79 ± 0.92^{b}	3.42 ± 0.72^{b}	3.13 ± 0.96^{b}

means (± SD) in the rows followed by the same letter are not significantly different according to the Duncan's *t*-test at *P* = 0.05; K40 – somaclone; Els. – cv. Elsanta; Sen. – cv. Senga Sengana; O – shoots micropropagated *in vitro* during 45 passages; N – plants propagated traditionally for 4 generations and then micropropagated for 8 passages

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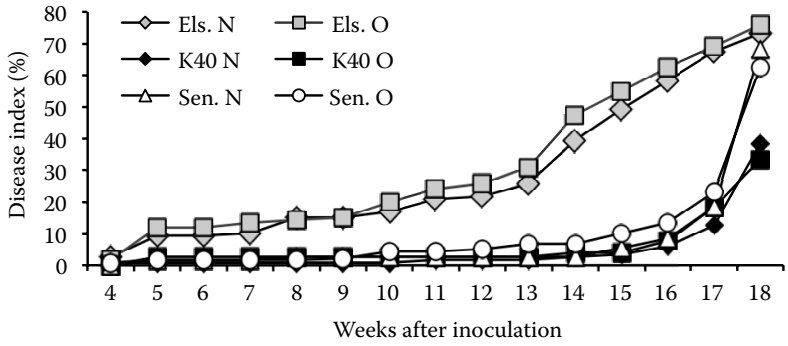

Fig. 1. Disease index of Verticillium wilt in strawberry genotypes challenged with *Verticillium dahliae* in the greenhouse test K40 – somaclone; Els. – cv. Elsanta; Sen. – cv. Senga Sengana; O – shoots micropropagated *in vitro* during 45 passages; N – plants propagated traditionally for 4 generations and then micropropagated for 8 passages

There were significant differences in susceptibility to *Verticillium dahliae* between plants belonging to the three genotypes tested. The first symptom of the disease such as loss of turgor was observed on the leaves of cv. Elsanta 3–4 weeks after infection. On the fifth week, yellowing and wilting occurred on the old leaves positioned on crown's periphery. Later on, the leaf blades turned brown and began dying out, starting from the older leaves towards the younger ones growing at the crown's centre. Since the 13th week the increase in infection rate and Verticillium wilt disease index accelerated and after 18 weeks from infection all the leaves on cv. Elsanta plants were diseased and many plants have died (Table 4, Fig. 1).

As opposite, on K40 and cv. Senga Sengana plants there were no visible symptoms of the disease up to the 9th week. The mild wilting on old leaves started to occur sporadically since the 10th week from infection, especially during sunny days, but leaf yellowing was observed as late as the 16th week. Since then, an apparent breakdown of resistance occurred in plants of both genotypes but the progress of disease was slower in plants of K40 clone that in cv. Senga Sengana (Table 4). On the 18th week from infection disease index of cv. Senga Sengana plants (*DI* = 65) was slightly lower than the index of cv. Elsanta (*DI* = 75) but the plants of K40 clone were in much better shape (*DI* = 35) (Fig. 1).

The two-factorial analysis of variance (genotype versus plant propagation method) has shown a strong effect of genotype and lesser of propagation method on Verticillium wilt disease index, but no significant interaction between the two factors (Table 5).

To fulfil the Koch's postulates, after completed trials, from plant tissues with disease symptoms the pathogen was isolated on selective medium for Verticillium according to Talboys (1960) and again identified as *Verticillium dahliae* based on macro- and microscopic appearance.

Somaclonal variation has been frequently used as a source of useful trait in strawberry breeding. Somaclones with enhanced resistance to *Phytophthora cactorum* (Battistini, Rosati 1991), *Fusarium oxysporum* f.sp. *fragariae* (Toyoda et al. 1991) and *Colletotrichum acutatum* (Hammerschlag et al. 2006) were selected and tested. Other useful traits derived from somaclonal variants include plant vigour, fruit yield and fruit colour (Fukuoka et al. 1996; Popescu et al. 1997; Biswas et al. 2009). However, only one somaclone, with enhanced resistance to *Alternaria alternata,* has been thus far registered as a new cv. Akita Berry in Japan (Takahashi et al. 1997).

The advantage of *in vitro* selection is that it speeds up breeding process. Somaclonal variation may generate traits that are not present in the ge-

Table 5. The two-factorial analysis of variance for the mean index of Verticillium wilt disease in strawberry genotypes

Source of variation	Degrees of freedom	Mean square	F_{emp}
Genotypes	2	15.11112	381.161**
Method of reproduction	1	0.21007	5.299*
Interaction	2	0.01796	0.453
Error	138	0.03964	

F_{emp} – Fisher's test empirical value; *significant differences between treatments at $P = 0.05$; **significant differences between treatments at $P = 0.01$

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netic pool of a given species. However, somaclonal variants may not be stable and heritable during sexual or even clonal propagation. Sansavini et al. (1990) reported that somaclonal variations occurring during micropropagation of strawberry were inherited during the first propagation from runners but subsequently declined in the next seasons. Similarly, Biswas et al. (2009) found that some of the somaclonal traits elicited in strawberry *in vitro* cultures reverted back to their original phenotype within 2–3 vegetative generations. Forni et al. (2001) demonstrated that changes in chromosome number occur not only in *in vitro* cultures but also during plant propagation from runners. However, in four of the five cultivars tested these variations were transient and have disappeared during further plant development in a greenhouse.

In our previous experiments we selected K40 somaclone of cv. Elsanta strawberry, which is highly resistant to Verticillium wilt (Sowik et al. 2004b; Sowik, Michalczuk 2007). In this experiment we have demonstrated that the main traits of this somaclone were retained during long-term *in vitro* shoot proliferation as well as during 4 generations of clonal propagation form runners followed by several *in vitro* subcultures. Irrespective of the method of propagation, the plants of the somaclone K40 strawberry were much more resistant to *Verticllium dahliae* in greenhouse test than the plants of the original cultivar and the plants of cv. Senga Sengana, which is considered as resistant to Verticillium wilt in field conditions. This proves that this trait elicited as somaclonal variant is stable and heritable both during *in vitro* micropropagation and clonal plant propagation from runners.

The fruit characteristics of the somaclone were not affected by selection but the plants were more vigorous than the plants of the original cultivar observed previously (Sowik et al. 2001, 2004a,b, 2007; Sowik, Michalczuk 2002). In the present experiment we proved that high plant vigour was stable during 45 generations of *in vitro* shoot proliferations as well as during 4 generations of clonal propagation from runners. Strawberry plants propagated *in vitro* had often higher growth vigour than those propagated from runners, but this trait is usually lost in the next seasons in the field (Cameron, Hancock 1986). However, the plants of K40 clone are more vigorous than the plants of the original cultivar irrespective of the propagation method. This shows that enhanced growth vigour is a stable trait which was co-selected with the resistance to *Verticillium dahliae*.

Our experiments demonstrated that somaclonal variation is a valuable source of disease resistance in strawberry breeding programmes. The selected somaclone is highly resistant to *Verticillium dahliae* and the trait is stable and heritable both during *in vitro* axillary shot proliferation and clonal propagation from runners.

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