A METHOD FOR *IN VITRO* TESTING STRAWBERRY SUSCEPTIBILITY TO VERTICILLUM WILT

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A B S T R A C T

A method was developed for evaluating strawberry susceptibility to Verticillium wilt in *in vitro* conditions. Micropropagated strawberry shoots of 15 strawberry cultigens, differing in susceptibility to the disease in field conditions, were treated *in vitro* with fungal homogenate of *Verticillium dahliae*, diluted with sterile water in a proportion of 1:10 in order to decrease the pathogen's pressure. The susceptibility was estimated on a 6-point ranking scale based on infection symptoms occurring on the shoots. The results obtained in *in vitro* test were consistent with the susceptibility of strawberry genotypes to Verticillium wilt in the field.

Key words: strawberry, *in vitro* testing, fungal homogenate, Verticillium wilt, susceptibility

INTRODUCTION

Verticillium dahliae is a causative agent of Verticillium wilt, being devastating in strawberry production (Amenduni et al., 2004). This pathogen is commonly present in soils, which makes difficult to control the disease through elimination of infection sources (Steventon et al., 2002; Mercado-Blanco et al. 2003; Porras Soriano et al., 2003; Uppal et al., 2008). Therefore, resistant cultivars of strawberry are sought after for planting in order to reduce the damages caused by the disease. However, at present only a few cultivated strawberry varieties are considered to be resistant or highly tolerant while the majority of new, desert cultivars are susceptible or very susceptible to *Verticillium dahliae* infection (Żurawicz et al., 2005). Their cultivation in fields infested with the

pathogen may lead to a complete devastation of plantation within few months after planting (Bielenin et al., 1998; Meszka et al., 2006; Meszka and Bielenin, 2007). Therefore, resistance/tolerance to Verticillium wilt is one of the most important traits taken into account in cultivation and in strawberry breeding programmes.

In traditional breeding, susceptibility/resistance to this disease of hybrids obtained is tested by planting them into fields heavily infested with pathogen and observation of disease symptoms (Aguado et al., 2008). This method is time-consuming and often affected by weather conditions. Therefore, usually two seasons are required to evaluate resistance of a given genotype (Nothmann and Ben-Yephet, 1979; O'Brien, 1983; Masny et al., 1999).

Biotechnology provides breeders with new techniques which widen the available genetic variability and speed up breeding process (McNicol and Graham, 1992; Qin et al., 2008). Among them, the main role in breeding for resistance plays the technology of plant DNA recombination and directed mutagenesis (Schestibratov and Dolgov, 2005; Debnath and Teixeira da Silva, 2007; Husaini and Abdin, 2008). However, the critical point in these techniques is the early selection of obtained variants/transformants. Since in plant biotechnology most of the manipulations are done on callus or organ cultures and the new plantlets are regenerated *in vitro*, for field testing they must be multiplied and acclimated to *ex vitro* conditions. This significantly lengthens up the whole process. Therefore, an efficient method for early testing/selection in *in vitro* conditions is of the utmost importance.

In vitro cultures of tissues, organs and shoots were often used to test crop plants susceptibility to infection with various fungal pathogens (Agnola et al., 2003; Vidal et al., 2004). For susceptibility testing, usually the filtrates of pathogens cultures, containing their phytotoxic metabolites as well as solution of pure toxins, synthetic or natural, were used (Czaplińska, 1978; Ireland and Leath, 1987; Rosati et al., 1989; Connell et al., 1990; Nachmias et al., 1990; Rosati et al., 1990; Płażek, 1994; Cristinzio et al., 1994; Koike et al., 1996; Palmer et al., 2005). In our previous experiments, the sterile filtrates of *Verticillium dahliae* cultures did not affect the growth and development of *in vitro* strawberry shoot cultures whilst the point-inoculation with the living pathogen mycelium caused inhibition of growth and subsequent death of shoots (Sowik et al., 2001).

The aim of the work presented was to develop the method for testing the strawberry susceptibility to *Verticillium dahliae* infection in *in vitro* conditions with the use of homogenate of living pathogen culture.

MATERIAL AND METHODS

Plant material

For experiments, strawberry plants (*Fragaria* x *ananassa* Duch.), cultivars 'Aga', 'Astra', 'Elkat', 'Elsanta', 'Filon', 'Kaster', 'Luna',

'Seal' 'Senga Sengana' and breeding clones K-1284, K-1299, K-1349, K-1356, K-1376 and K-1472, bred at the Department of Fruit Plant Breeding, Research Institute of Pomology and Floriculture in Skierniewice, Poland, were used. In the field experiments, these genotypes showed different level of susceptibility to Verticillium wilt (Masny et al., 1999).

The *in vitro* strawberry shoot cultures were established from runner tip meristems taken from virus-free mother plants, grown in a greenhouse. The composition of basal medium was taken from Boxus (1974). It was composed of Knop's (1865) macroelements, Murashige and Skoog's (1962) microelements and vitamins, glycine (2 mg l^{-1}), inositol (100 mg l^{-1}), glucose (40 g l^{-1}), IBA (0.5 mg l^{-1}), BA (0.5 mg l^{-1}), GA_3 (0.1 mg l^{-1}) and solidified with Difco bacto agar (8 g l^{-1}). pH of the medium was adjusted to 5.6-5.7 prior to autoclaving. The *in vitro* cultures were maintained in a growth chamber at 23ºC/18ºC (day/night), under white light with the quantum irradiancy 55 µmol m^{-2} s^{-1} and 16 h photoperiod. For experiments, the non-rooted strawberry shoots, obtained directly after dividing proliferated shoot clusters, were used.

Verticillium dahliae Kleb. cultures

Pathogenic fungi were isolated from strawberry plants with typical symptoms of Verticillium wilt, collected from experimental field of the Department of Fruit Plant Breeding, Research Institute of Pomology and Floriculture in Skierniewice. The field was used for many years for testing strawberry susceptibility to Verticillium wilt and the concentration of *Verticillium dahliae* in the soil is very high (Bielenin and Żurawicz, 1994). Fifteen isolates of *Verticillium dahliae* were identified on the basis of their macro- and microscopic appearance. For further use they have been stored as pure cultures on LBA medium at 5ºC in darkness. Before starting the testing experiment, inoculum of each isolate was transferred to new Petri dishes with LBA medium and cultured at 21ºC for 2 weeks. For susceptibility testing only well developed cultures, that covered completely the medium surface, were chosen. Rings of medium, 8 mm-in-diameter, overgrown with fungal mycelium, were cut out and transferred to 100 ml Erlenmeyer flasks containing 15 ml of liquid 2% Bacto Malt Extract medium. After 21 days of incubation at 21ºC, the cultures were homogenized (mycelium with the medium) for 90 s at 11000 rpm (Ultra-Turrax homogeniser, IKA Werke, Germany) and mixed. The mixed homogenate was diluted 10 times with sterile distilled water in order to reduce the pathogen's pressure and to enable the precise evaluation of differences in susceptibility to *Verticillium dahliae* infection between various strawberry genotypes.

For testing, strawberry shoots were transferred to the basal medium (6 shoots per 100 ml Erlenmeyer flask) and the 3 ml of diluted homogenate was

layered on the surface of the medium. The susceptibility was estimated on the basis of observation of infection symptoms occurring on the shoots. The evaluation was carried out after 28, 46, 56 and 76 days of co-culturing on a 6-point ranking scale, where:

0 – healthy shoots, no symptoms on leaves,
1 – shoots with single leaves showing infection symptoms (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 infected.

Shoots grown on the basal medium on which 3 ml of sterile liquid medium used for fungal pathogen culture were layered, served as a control. The experiment was performed in 3 replications, where one Erlenmeyer flask with 6 shoots constituted a replication. The results were statistically elaborated with analysis of variance followed by Duncan's "t" test for means separation at $p = 0.05$.

RESULTS

After 28 days of co-culturing with the pathogen, shoots of cultivars 'Elsanta' and 'Kaster' were the most severely damaged by the pathogen. The severe symptoms of disease had also shoots of cultivars 'Elkat' and 'Luna' and breeding selection K-1376. The least susceptible to infection with *Verticillium dahliae* was 'Senga Sengana' – only 33.3% of shoots had a few leaves with slight change in colour (Fig. 1).

After 46 days of experiment it was possible to distinguish 3 groups of strawberry genotypes that showed different levels of damage by the pathogen (Fig. 2). The first group, consisted of 'Elsanta' and 'Kaster', had the most severe symptoms of infection. Shoots of cultivars 'Elkat' and 'Luna' and of breeding clone K-1376 were less damaged and were assigned to the second group. The rest of genotypes showed significantly lower susceptibility to *Verticillium dahliae* infection, although among them there was also variability in susceptibility level that was clearly visible after 56 days from inoculation (Fig. 3). After this period of co-culturing strawberry shoots with *Verticillium dahliae*, the least infection symptoms among these genotypes were observed on 'Seal', 'Filon', K-1284 and 'Senga Sengana' and they were assigned to the group IV. The remaining ones (clones K-1472, K-1356, K-1349, K-1299 and cultivars 'Aga' and 'Astra') showed susceptibility significantly higher than these belonging to the group IV but lower than genotypes of the group II. Thus, they were clustered as the group III.

The longer maintenance of strawberry cultures with mycelium homogenate resulted in a high infection rate of all strawberry genotypes tested and the differences between them became less distinguishable (Fig. 4).

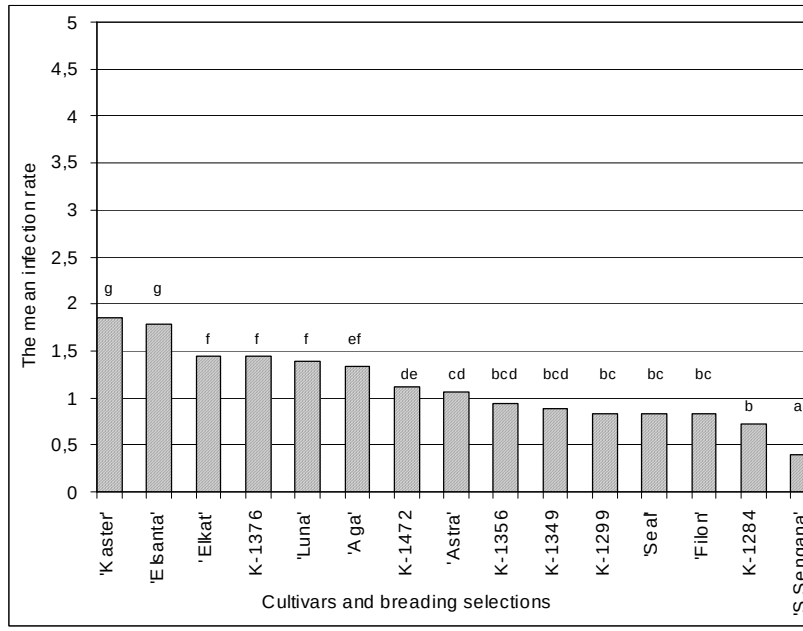

Figure 1. Infection rate of strawberry cultivars and breeding lines with *Verticillium dahliae* in *in vitro* conditions on 28th day of experiment. The bars on the diagram marked with the same letters don't significantly differ at $p \leq 0.05$ according to Duncan's "t" test

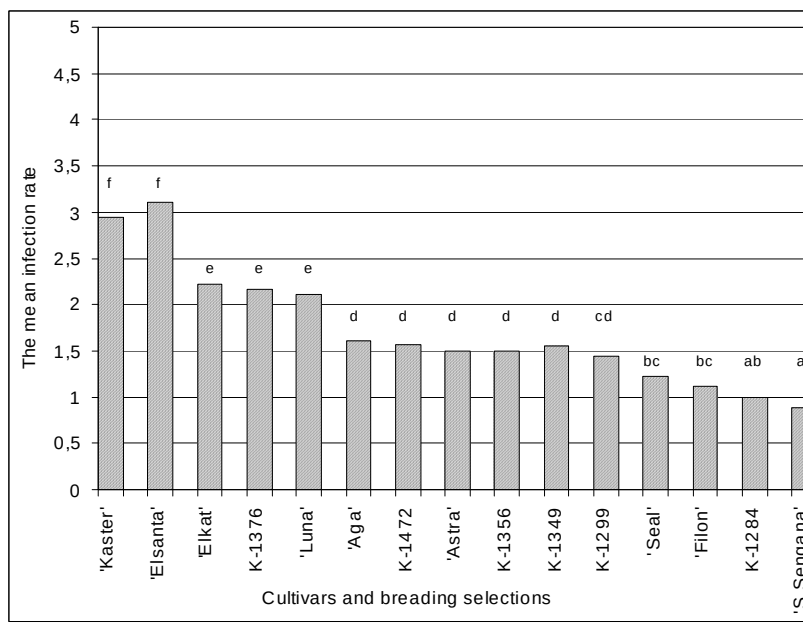

Figure 2. Infection rate of strawberry cultivars and breeding lines with *Verticillium dahliae* in *in vitro* conditions on 46th day of experiment

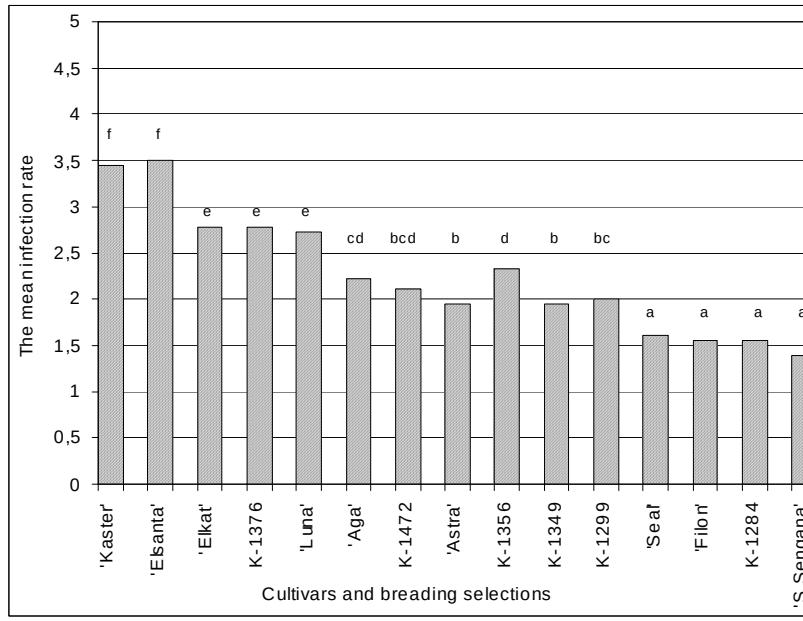

Figure 3. Infection rate of strawberry cultivars and breeding lines with *Verticillium dahliae* in *in vitro* conditions on 56th day of experiment

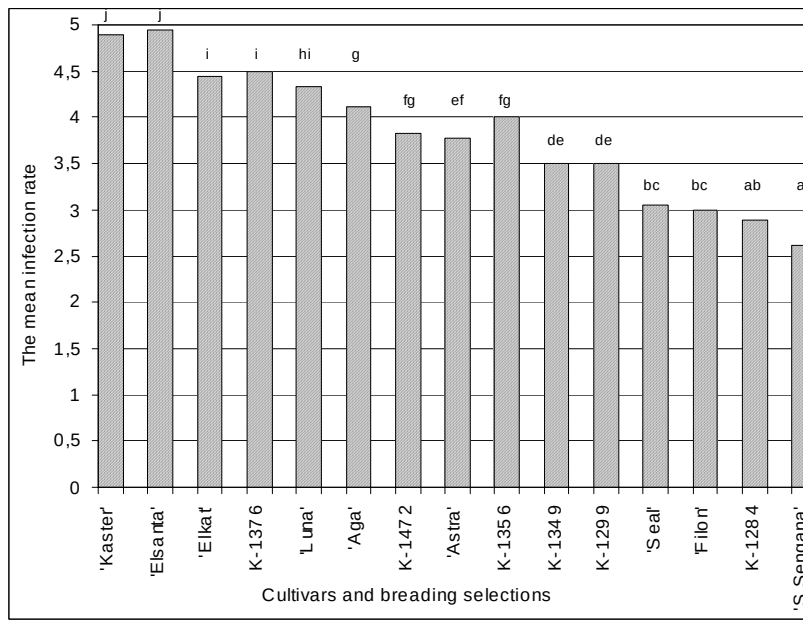

Figure 4. Infection rate of strawberry cultivars and breeding lines with *Verticillium dahliae* in *in vitro* conditions on 76th day of experiment

DISCUSSION

The reaction of the strawberry cultivars and breeding lines to *Verticillium dahliae* homogenate treatment in *in vitro* test was consistent with their susceptibility to Verticillium wilt in the field. Among genotypes tested the most susceptible to *Verticillium dahliae* infection in *in vitro* conditions were shoots of 'Kaster' and 'Elsanta' (the group I). These cultivars are also very sensitive to Verticillium wilt in the field (Masny et al., 1999; Żurawicz, 2005).

Cultivars 'Elkat', 'Luna' and breeding clone K-1376 were significantly less susceptible to *Verticillium dahliae* homogenate treatment than 'Kaster' and 'Elsanta' and were classified as group II. In the field tests carried out by Masny and co-workers (1999) these genotypes showed medium susceptibility to the disease. Both 'Elkat' and breeding selection K-1376 originated from cultivar 'Elsanta', which is very susceptible to *Verticillium dahliae* (Masny et al., 1999).

Still less sensitive to treatment with pathogen's homogenate *in vitro* were breeding clones K-1299, K-1349, K-1356 and K-1472 and cultivars 'Aga' and 'Astra' (the group III). They were marked as genotypes of a low susceptibility to Verticillium wilt also in the field tests conducted by Masny et al. (1999). These clones derived from Polish cultivars, highly resistant to the disease (Masny et al., 1999; Żurawicz, 2005).

Cultivar 'Senga Sengana' as well as 'Seal', 'Filon' and breeding clone K-1284, which are progenies of highly resistant cultivars 'Senga Sengana' and 'Dukat' (Rebandel, 1993; Żurawicz and Bielenin, 1995; Masny et al., 1999; Żurawicz, 2005; Meszka et al., 2006), showed the least susceptibility to *Verticillium dahliae* in *in vitro* conditions. This was consistent with results obtained in the field tests by Masny et al. (1999).

As opposed to the field tests, the elaborated method enables quantitative evaluation of susceptibility level of strawberry genotypes to *Verticillium dahliae* infection. In the 6-point scale, the susceptibility level of genotypes tested in standard conditions (i.e. duration of the test – 56 days, the mycelium homogenate diluted 10 times) was: for the group I > 3.5, the group II – 2.51-3.5, the group III – 1.51-2.5 and the group IV – 0-1.5. Such quantitative data are highly important in screening for markers of resistance genes, studying their inheritance and genome mapping.

Pathogen prepared as a homogenate was indicated as a very effective and convenient inoculum (Sowik et al., 2001). In this experiment, *Verticillium dahliae* applied to the strawberry shoot culture as mycelium homogenate infected strawberry shoots uniformly and the results were consistent in several replications. This technique enables to use many isolates at the same time in the test, which is desirable due to differences in the virulence level between various pathogen isolates (Leski, 1974; Madhosingh, 1995; Agnola et al., 2003; Mercado-Blanco et al. 2003; Uppal et al., 2008).

Homogenate of *Mycosphaerella musicola* mycelium was applied by Trujillo and Garcia (1996) for evaluating banana cultivars susceptibility to Yellow Sigatoka leaf spot disease. In their study, the results of *in vitro* tests were also correlated with banana plants susceptibility to the pathogen in the field.

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TESTOWANIE PODATNOŚCI ODMIAN I LINII HODOWLANYCH TRUSKAWKI NA WERTYCYLIOZĘ W WARUNKACH *IN VITRO*

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S T R E S Z C Z E N I E

Opracowano metodę testowania podatności truskawki na wertycyliozę w warunkach *in vitro*. Mikrorozmnażane pędy 9 odmian i 6 klonów hodowlanych truskawki (otrzymane bezpośrednio po rozdzieleniu namnożonych wielopędów), różniących się podatnością na tę chorobę w warunkach polowych, traktowano homogenatem kultury *Verticillium dahliae* rozcieńczonym sterylną wodą w stosunku 1:10 w celu zmniejszenia presji patogena i uchwycenia różnic odmianowych w podatności truskawki na porażenie. Podatność na chorobę oceniano w 6-stopniowej skali bazującej na objawach infekcji na pędach. Wyniki oznaczeń *in vitro* były zgodne z podatnością odmian i linii hodowlanych na wertycyliozę oznaczoną w warunkach polowych.

Słowa kluczowe: homogenat, odporność, podatność, testowanie *in vitro*, truskawka, wertycylioza, *Verticillium dahliae*