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In Vitro Selection and Somaclonal Variation in Alfalfa Verticillium Wilt.

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To obtain alfalfa genotypes with resistance to Verticillium wilt, *in vitro* selection using culture filtrate was conducted and the availability of this method was discussed. 20 regenerated plants were obtained from culture tissue which was derived from cotyledons of alfalfa cv. Kitawakaba (susceptible to Verticillium wilt) and selected for resistance to *Verticillium albo-atrum* culture filtrate. Of these, only 9 plants (45%) showed resistance to *V. albo-atrum* culture filtrate. Furthermore, 4 out of the 9 culture filtrate-resistant plants were susceptible to *V. albo-atrum* conidia inoculation. On the other hand, 11 culture filtrate-resistant plants were obtained in regenerants derived from non-selected callus tissue. These plants showed stable resistance to both *V. albo-atrum* culture filtrates and the conidia.

Introduction

Many reseachers have been trying to produce disease resistant plants using tissue culture techniques[1]. The methods most commonly used are *in vitro* selection with selective agents and utilization of somaclonal variation[2] without *in vitro* selection. The selective agents used for *in vitro* selection are crude culture filtrates of the pathogen[3,4] and partially or completely purified toxins, etc[5,6]. Somaclonal variation is an expression of genetic variability of plants regenerated from somatic cells via tissue culture[2]. In many plant species, disease resistant plants have also been produced by somaclonal variation without selective agents[2,7,8].

Verticillium albo-atrum Reinke *et* Bert. is a causal agent of Verticillium wilt of alfalfa[9]. This wilt disease occurs throughout Europe, the United States and Canada, and in the last decade also in Hokkaido, Japan[10]. The damage done by this disease gives rise to severe problems for production and durability of alfalfa sward. Resistant alfalfa cultivars which are appropriate for Japan have not yet been bred.

The purpose of this research is to obtain regenerant plants with resistance to Vertilillium wilt, using *in vitro* selection, from a cultivar grown in Hokkaido, Japan, and to estimate the efficiency of *in vitro* selection.

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Materials and methods

Callus initiation and suspension culture

Alfalfa (*Medicago sativa* L. cv. Kitawakaba) was used in this experiment. The calli were initiated from cotyledons, which turned out to be susceptible genotypes, on a medium of SH[11] supplemented with 2 mg/*l* 2, 4-D, 0.2 mg/*l* Kinetin and 0.8% agar in an incubator at 25 ±1°C. After one month, suspension cultures were obtained by transferring friable calli lumps to a liquid medium of the same composition as that of callus initiation. After the density of suspension cells increased, selection was initiated.

Selection procedure and plant regeneration

The pathogen (*Verticillium albo-atrum* Reinke *et* Bert., abbreviated as Vaa) was supplied by Mr. R. Sato (the Hokkaido National Agricultural Experiment Station, Japan). To ensure the persistence of virulence, alfalfa plants were inoculated with the fungus, after which, the virulent fungus was again isolated. The pathogen was kept at 20°C in Potato Sucrose Agar[12].

A tip of fungal colony mat was then placed in a 500 m*l* Sakaguchi flask with 200 m*l* of Czapek -Dox broth[12] and cultured for 3 weeks on a rotary shaker. The medium was filtered first through a triple layer of gauze, then through a filter paper and finally sterilized with a filter with pore size of 0.45 μm. Undiluted culture filtrate was used to prepare selective media, which consisted of SH broth containing 2 mg/*l* 2, 4,-D, 0.2 mg/*l* kinetin and 10% (v/v) culture filtrate. Alfalfa suspension culture was transferred to the selective medium (PCV 3.3%: 1.0 m*l* of suspension cells to 29 m*l* of selective medium), and the first selection was performed on a rotary shaker for the following two weeks. Then, 1.0 m*l* of the cells washed three times with 29 m*l* of fresh SH liquid medium were plated on SH agar medium (2 mg/*l* 2, 4-D, 0.2 mg/*l* kinetin). After one month of culture, the recovered calli were transfered to SE5K[13] medium (SH+5 mg/*l* 2, 4-D, 2 mg/*l* kinetin) containing Vaa culture filtrate (10% v/v) in petri dishes (90×15 mm) to exclude escapes, and the second selection was carried out for one month by incubating at 25 ±1°C in the dark. The calli grown on the selective medium were then transferred to modified hormone free SH medium containing 50 mM proline, 22.4 mM (NH_4) SO_2 and 0.8% agar[13] to induce somatic embryos. After the formation of somatic embryos, they were transferred to 1/2 (conc.) SH to allow the production of plantlets. To estimate the efficiency of *in vitro* selection, the same procedure was done without fungal culture filtrate.

Inoculation of pathogen and evaluation of resistance.

The regenerated plants were placed in 20 cm-diam. wagnel pots with soil and grown under greenhouse conditions. When the plants reached a height of 20 cm, inoculation with the pathogen was initiated. To produce Vaa conidia, the pathogen was incubated in Potato Sucrose Broth[12] in 200 m*l* Sakaguchi flasks for one week at 25 ±1°C. The method of preparation of Vaa culture filtrate was described above. Two inoculation methods were applied. One was culture filtrate stem-cutting assay and the other was leaf inoculation with Vaa conidia. Stem cutting assay was carried out as follows. (1) Stem cuttings from plants (5 stems per plant) were placed in test tubes containing 10 m*l* of 1 : 19 Vaa culture filtrate/distilled water. (2) The treated stems were kept in an incubator at 20 ±1°C and under illumination of 2,000 lux for three days. The cuttings were rated on a scale of 0 (no symptom)-5 (wilted wholly). The score for each plant was calculeted as the average of the ratings of the 5 stems.

The following procedures were adopted for leaf inoculation: (1) Five leaves of each plant were cut by a sterilized cutter. (2) These leaves were dipped in fungal suspension of 1×10^6 conidia/m*l*

for 30 minutes, and (3) placed on wet filter paper in a petri dish. After one week, the inoculated leaves were rated on a scale from 0-5 according to the visual symptoms of the leaves (0: green and no chlorosis～ 5: whole chlorosis).The score of each plant was calculated as the average of the rating of the 5 leaves. In each inoculation method, 50 plants of cv. Kitawakaba were also included as controls.

Results and discussion

The number of recovered calli during *in vitro* selection processes are shown in **Fig. 1**. The number of surviving calli after the first selction porcedure was less than 20% that of control. In the second selection, 35. 9% (47 calli) of selected calli could be grown on selective media and a few calli were regenerated from these selected calli. Since selected and non-selected regenerable calli were derived from the same explant, we could estimate the efficiency of *in vitro* selection.

Fig. 2 shows the comparison of the distribution of wilting scores in regenerated plants from the calli with or without culture filtrate selection to that of the control. Average wilting scores of the 50 plants of the original cultivar, those regenerated from the calli resistant to culture filtrate, and from non-selection calli were 2. 18±0. 16, 1. 74±0. 33 and 2. 17±0. 26, respectively. The difference in wilting scores among, plants of the original cultivar and both regenerants was not statistically significant, indicating that the selected regenerants were not more resistant to Vaa culture filtrate than the non-selected regenerants or original cultivar.

Fig. 3 illustrates the distribution of leaf symptom scores of leaf inoculation with Vaa conidia in the three populations. Average scores of the regenerants from calli with or without culture filtrate selection and 50 plants of original cultivar were 1. 88±0. 12, 1. 81±0. 21 and 2. 15±0. 17, respectively. As in the case of the wilting scores, the differences among the average scores were not significant. These results indicate that the selected regenerants were not more resistant to Vaa conidia than the non-selected regenerants or original cultivar.

Table 1 shows the responses of the culture filtrate-resistant plants with a wilting score of less than 1. 0 to the inoculation of Vaa conidia to the leaves. Selected regenerant cell lines 313-2-5, 313-2-12,

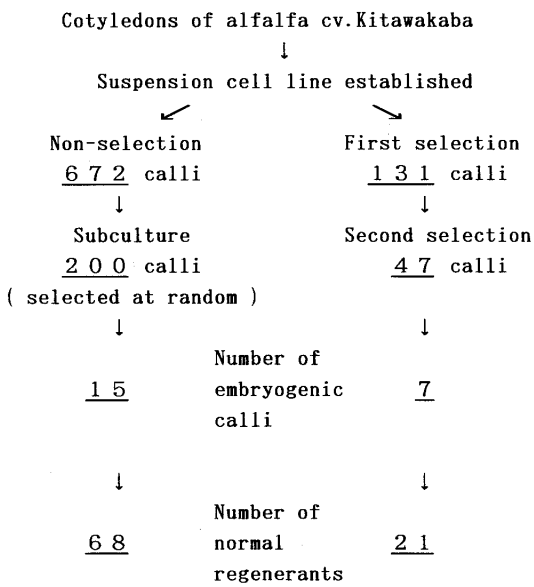

Fig. 1 *In vitro* selection scheme and numbers of recovered calli and plants.

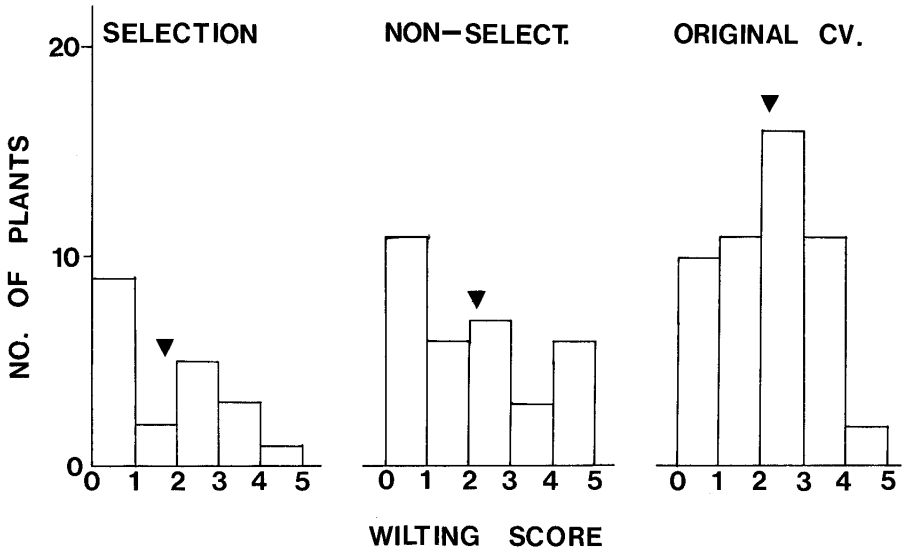

Fig. 2 The distribution of wilting scores of alfalfa (original cv Kitawakaba) regenerants from selected and non-selected callus in response to *Verticillium albo-atrum* culture filtrate. Each score was based on six-point severity scale where 0=lowest and 5=highest. ▼ indicates mean value.

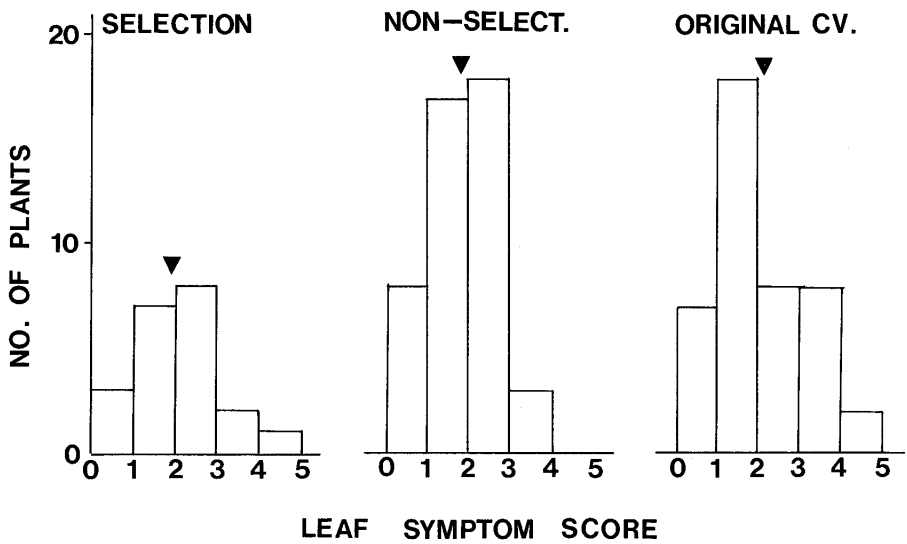

Fig. 3 The distribution of leaf symptom scores of alfalfa (original cv. Kitawakaba) regenerants from selected and non-selected callus in response to *Verticillium albo-atrum* conidia. Each score was based on six-point severity scale where 0=lowest and 5=highest. ▼ indicates mean value.

313-2-15 and 313-2-30 showed resistance to Vaa culture filtrate but not to Vaa conidia. However, non-selected regenerants with resistance to culture filtrate were almost resistant to the pathogen. A similar result was previously obtained by Toyoda *et al*[14]. They developed bacterial wilt-resistant tomato plants through tissue culture with or without *in vitro* selection using a culture filtrate of *Pseudomonas solanacearum* as a selective agent. Selected regenerants expressed bacterial wilt resistance at an early infection stage, but wilted at a later stage. In contrast, resistant somaclonal variants under the non-selection scheme showed more stable resistance.

Table 1. Verticillium wilt resistance of alfalfa regenerants which had low wilting score less than 1.0 to *V. albo-atrum* culture filtrate.

Treatment	Callus lines	Wilting score[a]	Leaf symptom score[b]
CF-selection[c]	313-1-2	0.2	0.5
	313-1-8	0.6	1.3
	313-2-5	0.4	2.5
	313-2-12	0.0	2.9
	313-2-15	0.4	3.1
	313-2-22	0.4	1.6
	313-2-23	0.2	0.5
	313-2-29	0.4	1.0
	313-2-30	1.0	2.5
Non-selection	K3C-1-3	1.0	0.3
	K3C-1-5	1.0	1.4
	K3C-1-8	0.0	1.3
	K3C-2-3	0.8	1.1
	K3C-2-5	0.0	0.3
	K3C-2-6	0.8	0.5
	K3C-2-8	1.0	0.4
	K3C-2-12	0.8	1.2
	K3C-3-4	0.4	0.9
	K3C-3-9	0.9	1.2
	K3C-4-13	0.5	0.3

a, b: each score was based on six-point severity scale where 0=lowest and 5=highest. c: *Verticllium albo-atrum* culture filtrate selection.

It is known that *Verticillium albo-atrum* produces wilting toxin,[15,16] but host-specific toxin has not been identified. Latunde-Dada and Lucas[17] used the low molecular weight toxin extracted from Vaa as the selective agent for producing disease resistant alfalfa plants. But selected plants failed to transfer the resistance into their progenies. In another case[3], crude culture filtrate was also shown to be effective as a selective agent in this disease. However, it was not determined whether the progenies of selected regenerants were resistant or susceptible. The present results suggest that the non-selected regenerants have relatively stable resistance to the pathogen. *In vitro* selection by using crude culture filtrate as a selection agent was not effective for producing Verticillium wilt resistant plants in our case. For obtaining resistant plants by applying *in vitro* selection methods, it is necessary to pursue research on *in vivo* as well as *in vitro* resistant mechanisms to this wilt disease.

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《和文要約》

アルファルファ・バーティシリウム萎ちょう病に対する細胞選抜とソマクローナル変異

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アルファルファ・バーティシリウム萎ちょう病菌(*Verticillium albo-atrum*)の培養ろ液を用いて、感受性品種キタワカバの培養細胞を選抜した．培養ろ液抵抗性カルスから再生した植物体の45.0%（20個体中9個体）が培養ろ液に対して抵抗性を示した．しかし，培養ろ液抵抗性植物体9個体中4個体は病原菌の分生胞子接種試験に対して抵抗性を示さなかった．一方，無選抜カルス由来植物体のうちで，培養ろ液に対して抵抗性を示した個体は分生胞子接種試験においても安定した抵抗性を示した．

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