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Alfalfa-*Verticillium albo-atrum* Interactions

IV. Reactions of alfalfa protoplasts to fungal culture filtrates and cell wall components

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Synopsis

KOIKE M., T. SHIMADA and Y. AMEMIYA (1993) : Alfalfa-*Verticillium albo-atrum* interactions IV. Reactions of alfalfa protoplasts to fungal culture filtrates and cell wall components. *J. Japan. Grassl. Sci.* **39**, 101-107.

Reactions of alfalfa protoplasts of six genotypes (three resistant and three susceptible genotypes) to *Verticillium albo-atrum* culture filtrates fractionized by different molecular cut-off (3,500, 12-14,000 and 50,000) and to the cell wall components were investigated. Protoplasts derived from susceptible genotypes reacted to a low molecular weight fraction (3,500 <) more highly than those derived from resistant genotypes. On the other hand, protoplasts derived from resistant genotypes reacted to high molecular weight fractions 12-14,000 ~ 50,000, > 50,000) and *V. albo-atrum* cell wall components more highly than those from susceptible genotypes. These results indicate that low molecular weight fractions may be useful for *in vitro* selection using *V. albo-atrum* culture filtrate, and that the percentage of reacted protoplasts to the fungal cell wall components may be a good indicator for the *in vitro* resistance.

Key words : Alfalfa, Cell wall components, Culture filtrate, Protoplast, *Verticillium albo-atrum*.

Introduction

Verticillium albo-atrum Reinke *et* Berthold is a causal agent of Alfalfa Verticillium wilt[1]. This wilt disease occurs throughout Europe, United States and Canada, and in Hokkaido, Japan as well[10]. The damage by this disease gives rise to severe problems for production and durability of alfalfa sward. Control of this disease has largely been depending on the induction of resistant cultivars.

Plant tissue culture systems have been considered as potential sources for the breeding for novel resistance which may be selected *in vitro*. In alfalfa Verticillium wilt, some researchers reported *in vitro* selection[6,11,16,18]. A great deal of effort has been made on breeding Verticillium wilt-resistant alfalfa plants through tissue culture techniques. What seems to

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be lacking, however, is a research for a correlation between *in vivo* resistance and *in vitro* reaction to cytotoxic components of *V. albo-atrum* culture filtrates.

For the studies on host-parasite relationship, protoplasts have been used as a simplified system in various plant disease[3,4,7,8,9,14]. This method is available to observe the host-parasite interaction in controlled environments and gives good reproduction of the results. In a previous study[14], we reported that there was a correlation between *in vivo* resistance (cultivar level) and *in vitro* (protoplast level) reaction to low and high molecular weight fractions of *V. albo-atrum* culture filtrates. But there was no correlation between *in vivo* resistance and *in vitro* reaction to the non-fractionized culture filtrates.

The aim of this research is to study the possible correlation between *in vivo* resistance of alfalfa genotypes and *in vitro* responses (protoplast level) to subdivided fractions of the culture filtrates and *V. albo-atrum* cell wall components.

Materials and Methods

1. Biological materials

Six alfalfa (*Medicago sativa* L.) genotypes were used ; three resistant genotypes (V-16, E 18 and K-1 derived from cvs. 'Vertus', 'Europe' and 'Kitawakaba', respectively) and three susceptible genotypes (V-6, E-8 and T-3 derived from cvs. 'Vertus', 'Europe' and 'Thor', respectively) to Verticillium wilt. The resistance level of each genotype is shown in Table 1. An isolate of *V. albo-atrum* was obtained from Mr. R. Sato, Hokkaido National Agricultural Experiment Station, Japan.

2. Callus initiation and protoplast isolation

Callus initiation[13] and protoplast isolation[14] were made as described previously.

3. *V. albo-atrum* culture filtrates production and fractionations

Production of culture filtrate and its fractionation were carried out as described previously[14]. The culture filtrate (CF) was separated into four fractions using dialysis tubes with molecular cut-off 3,500, 12-14,000 and 50,000. The fractions of 3,500 <, 3,500 ~ 12-14,000 12-14,000 ~ 50,000 and > 50,000 were abbreviated as A, B, C and D, respectively.

Table 1. Verticillium wilt resistance of six alfalfa genotypes used in this experiment.

Genotype	(cv.)	Wilting score[a]
V-16 (R)[b]	Vertus	0.2±0.1
E-18 (R)	Europe	0.3±0.2
K-1 (R)	Kitawakaba	0.5±0.1
V-6 (S)	Vertus	3.8±0.3
E-8 (S)	Europe	4.7±0.2
T-3 (S)	Thor	4.8±0.1

a : Wilting score was based on six-point severity scale where 0=lowest and 5=highest with inoculated *Verticillium albo-atrum* conidia (KOIKE *et al*[11]).
b : R=resistant, S=susceptible
±indicates standard error (n=15).

4. Preparation of *V. albo-atrum* cell wall components

The fungal cell wall components were prepared as described previously[12].

5. Effect of the fungal culture filtrates and cell wall components on protoplasts

Protoplast viability was assessed by exclusion of the dye Evans' Blue[5]. Protoplast preparations with a viability of > 90% were employed and their density was adjusted to 1×10^6/ml. Each fraction was added to protoplast suspensions at 20% based on SH[17] medium supplemented with Mannitol 13%. *V. albo-atrum* cell wall components (10 μg/ml as glucose equivalent) were added to the same SH medium. The protoplast death was recorded after incubation for 12 hours in the case of treatment with the clture filtrate fractions, after 3, 6, and 18 hours in the case of the fungal cell wall components. The protoplast density was also counted using a haemacytometer. In this experiment, the decreasing protoplast density means the increase in the percentage of collapsed protoplast, because the number of the collapsed protoplast could not be counted directly. All treatments were performed in triplicate and were repeated once to give 5 sample readings, each of > 100 protoplast counts in the case of protoplast viability. The percentages of protoplast death and density as a result of each fraction treatment were adjusted by the respective values obtained for control SH medium of treatment after the same period (typically this was 5–10% in each observation).

Results

1. Protoplast reactions to *V. albo-atrum* culture filtrate fractions

The percentage of protoplast viability to each fraction of *V. albo-atrum* CF is shown in Table 2. In a fraction A, protoplast viabilities of resistant genotypes were higher than those of susceptible genotypes. On the other hand, in fractions C and D, protoplast viabilities of resistant genotypes were lower than those of susceptible genotypes. The protoplast viability difference between susceptible and resistant genotypes was not significant in the total culture filtrates and fraction B. The difference was significant at fractions A, C and D.

Table 2. Percentages of alfalfa protoplast viabilities to *Verticillium albo-atrum* culture filtrates fractionized by dialyses.

Genotype	Fraction Molecular Weight (Da)				
	NF[a]	A (<3,500)	B (3,500～12-14,000)	C (12-14,000～50,000)	D (50,000>)
Resistant					
V-16	60.3± 7.8	71.9± 8.5	56.5±13.4	45.6±12.3	36.5± 9.8
E-18	58.5± 9.0	82.6±11.4	52.4± 9.6	54.5± 8.5	44.3±11.3
K-1	63.9±11.3	77.5± 7.9	60.4±15.6	48.6± 9.8	51.6± 8.9
Susceptible					
V-6	54.7± 9.6	52.4± 9.3	58.7±11.4	78.5± 6.8	82.3± 9.3
E-8	54.6± 7.8	46.8± 9.1	46.7± 8.9	75.7± 7.8	78.5±12.3
T-3	57.8± 8.8	48.9±10.6	51.5±12.5	82.6±11.6	80.5± 9.5
LSD (0.05)	6.63	14.10	16.26	16.23	9.14

a : Non-fractionized culture filtrates. ±indicates SD.

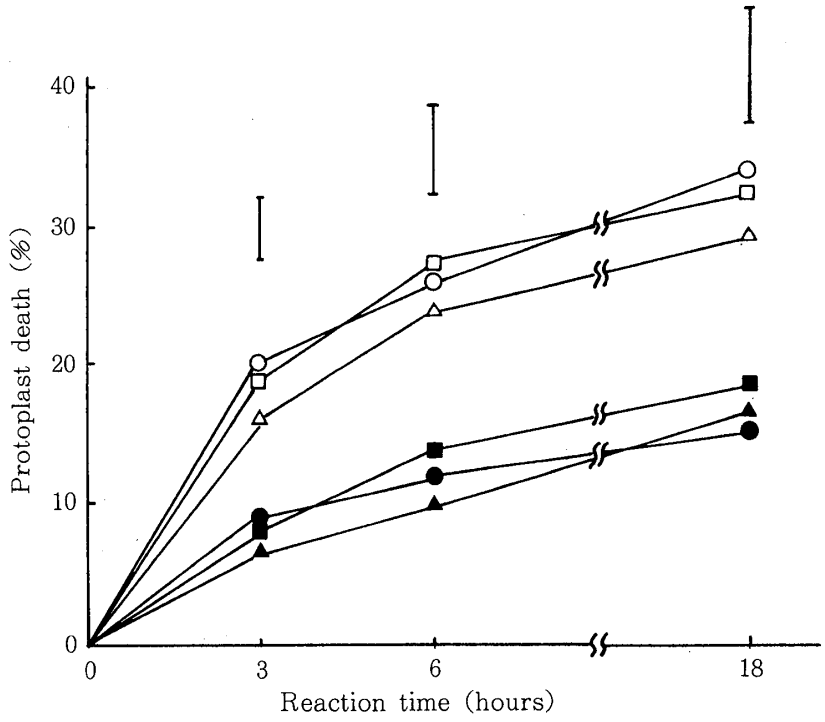

Fig. 1. The time course reactivity of protoplasts isolated from calli of six alfalfa genotypes against *Verticillium albo-atrum* cell wall components.
Resistant genotypes, ○ : V-16, □ : E-18, △ : K-1.
Susceptible genotypes, ● : V-6, ■ : E-8, ▲ : T-3.
Vertical bars indicate L.S.D. (p = 0.05).

2. Protoplast reactions to *V. albo-atrum* cell wall components

The time course of protoplast death rate *V. albo-atrum* cell wall components is shown in Fig. 1. The protoplast death rate of each genotype increased after treatment with *V. albo-atrum* cell wall components. The percentage of death protoplast of three resistant genotypes was higher (about twice) than those of susceptible genotypes in each reaction time. The difference in each reaction time was significant.

Discussion

Reactions of alfalfa protoplasts from six genotypes to fractions A, C and D were genotype-specific. In a fraction A, protoplasts of three susceptible genotypes (V-6, E-8 and T-3) reacted more highly than those of three resistant genotypes (V-16, E-18 and K-1). This result indicates that genotype-specific cytotoxic activity components or toxin may act to the susceptible protoplasts. The low molecular weight fraction (< 3,500 Da) may contain this toxin. In a previous study[14], a low molecular weight fraction of *V. albo-atrum* culture filtrates contained susceptible cultivar-specific activity. This was true for the case of the hop-*V. albo-atrum* interactions using low molecular weight fraction (< 5,000 Da) by a gel filtration reported by CONNEL *et al*[2].

The heat-released fungal cell wall components or fungal metabolites are known to be genotype- or cultivar- specific elicitors of disease resistant reactions (hypersensitive cell

death and phytoalexin production)[3,4,7,8,9,12,13]. In protoplast reactions to the fractions C, D and *V. albo-atrum* cell wall components, the protoplasts derived from three resistant genotypes reacted more highly than those from three susceptible genotypes. These results indicate that genotype-specific cytotoxic activity components or elicitors may induce the hypersensitive cell death of the protoplasts[3,4]. Recently, KOIKE *et al*[15]. proved that these high molecular weight fractions inducecd phytoalexin production in alfalfa calli.

In a previous study[13], alfalfa calli derived from three genotypes (different degrees of resistance to *Verticillium albo-atrum*) were treated with *V. albo-atrum* cell wall components. The result was that peroxidase and phenylalanine ammonia-lyase activities in callus extsacts were genotype-specific reaction. It may be concluded, therefore, that the percentage of reacted protoplasts to the elicitor determines the level of resistance.

Finally, from our results, in *in vitro* selection for alfalfa Verticillium wilt using fungal toxic metabolites, a low molecular weight fraction may be shown to be a good selective agent. On the other hand, the percentage of protoplasts reacted with the cell wall components (elicitor) may be a good indicator for resistance. However, the protoplasts used in our experiments were obtained from alfalfa calli. Therefore the further investigation with the protoplasts from foliar tissue also will be requested.

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アルファルファと *Verticillium albo-atrum* の相互作用

IV. 菌培養濾液および菌体細胞壁成分に対するプロトプラストの反応

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要 約

バーティシリウム萎ちょう病に対するアルファルファの細胞レベルにおける反応を明らかにするため，抵抗性3個体（V-16, E-18, K-1 それぞれ品種バータス，ヨーロッパ，キタワカバ由来），感受性3個体（V-6, E-8, T-3 それぞれ品種バータス，ヨーロッパ，ソア由来）より誘導したカルスからプロトプラストを調製し，*Verticillium albo-atrum* 培養濾液および菌体細胞壁成分に対する反応を調べた。

菌培養濾液を透析により4つの画分（A：分子量3500以下，B：3500以上12-14,000以下，C：12-14,000以上50.000以下，D：50,000以上）に分画し，それぞれのプロトプラストに20%の濃度で処理し，12時間後に生存率を調査した。各画分ともプロトプラストの生存率は減少したが，画分Aに対して感受性個体のプロトプラストの生存率の減少が顕著であり，画分C, Dに対しては抵抗性個体のプロトプラストの生存率の減少が顕著であった。

次にプロトプラストに菌体細胞壁成分を処理し，生存率を経時的に観察した。その結果，抵抗性個体のプロトプラストの生存率減少が感受性個体のそれらに比べ顕著であった。

以上の結果から，*Verticillium albo-atrum* 培養濾液を用いて細胞選抜を行う場合は，培養濾液全画分を選抜因子として用いるよりは，低分子画分（画分A）を分画して用いた方が効果的であることが予想される。また，培養濾液高分子画分（画分C, D）および菌体細胞壁成分に対するプロトプラストの反応率は抵抗性の指標として利用できることが示唆された。

キーワード：アルファルファ，細胞壁成分，培養濾液，*Verticillium aibo-atrum*，プロトプラスト．

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