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Alfalfa-*Verticillium albo-atrum* Interactions: III. Reactions of Protoplasts of Alfalfa Cultivars to Cytotoxic Components of Fungal Culture Filtrates

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Introduction

Verticillium albo-atrum Reinke *et* Bert. is a causal agent of Alfalfa Verticillium wilt[1]. This wilt disease occurs throughout Europe, the United States and Canada, and in a last decade also in Hokkaido, Japan[2]. The damage done by this disease gives rise to severe problems for production and durability of alfalfa sward. Control of this disease has been largely dependent on the breeding of resistant cultivars[3].

Plant tissue culture systems have been considered as one of the potential sources to develop novel resistance which may be selected *in vitro* of resistant clones. In alfalfa Verticillium wilt, a number of researchers reported *in vitro* selection using cytotoxic components of *V. albo-atrum* culture filtrates or crude culture filtrates. Suginobu *et al.*[4] and Frame *et al.*[5] showed that the *in vitro* selection for this wilt disease were effective, although Latunde-Dada & Lucas[6], and Koike *et al.*[7] reported negative results. Thus a great deal of effort have been made in breeding Verticillium wilt-resistant alfalfa plants through tissue culture techniques. What seems to be lacking, however, is research into the correlation between *in vivo* resistance and *in vitro* reaction to cytotoxic components of *V. albo-atrum* culture filtrates.

In a previous study[8], we reported that there was no correlation between *in vivo* resistance to Verticillium wilt and *in vitro* (callus level) reaction to *V. albo-atrum* culture filtrates. The aim of this research is to study the possible correlation between *in vivo* resistance of alfalfa cultivars and *in vitro* (protoplast level) responses to fungal culture filtrates.

Materils and Methods

Plant material. The alfalfa cultivars used in this experiment were 'Vertus' and '5444', highly resistant to *V. albo-atrum*, and 'Kitawakaba', 'Europe' and 'Thor', susceptible to it. The Alfalfa cultivars consisted of a heterogenic population. Therefore, the resistance levels of these cultivars were shown by percentages of resistant plants (**Table 1**).

Protoplast isolation. Cotyledon protoplasts were isolated from about 100 seedlings grown for 2 weeks *in vitro*. This leaf material (1.0 g) was incubated on an orbital shaker (50 rev/min) at 30°C

Table 1. Verticillium wilt resistance of 5 alfalfa cultivars used in this experiment.

cv.	n	Disease index[a]	Resistant plants (%)[b]
Vertus	115	1.4±0.3	83.5
5444	103	1.6±0.2	80.6
Kitawakaba	112	2.2±0.2	52.7
Europe	98	2.7±0.2	43.9
Thor	105	2.9±0.3	35.2

a: Disease index : 1=no symptom, 5=plant dead. Inoculation method was based on Sato[3]. ± indicates standard error.

b: Percentage of resistant plants showing low disease index (1 or 2).

in 10 m*l* of enzyme mixture (Cellulase RS, 10.0 g/*l*; Driselase 5.0 g/*l*; Pectolyase Y23 0.5 g/*l*; CPW salts with 13 % mannitol, pH 5.8). After cell wall digestion for 5 hours, protoplasts were pelleted by centrifugation (40×g, 5 min) and washed three times in CPW salts with 13 % mannitol.

Fungal isolate and preparation of culture filtrate. *Verticillium albo-atrum* isolated from alfalfa was obtained from Mr. R. Sato, Hokkaido National Agricultural Experimental Station, Japan and maintained on fresh Potato Sucrose Agar (PSA) at 20°C in the dark. One disc taken from 3-weeks culture on PSA was used to inoculate 200 m*l* of liquid Czapek-Dox broth. Cultures were shaken on an orbital shaker at 90 rev/min at 20°C for 3 weeks. Culture filtrates (CF) were prepared by filtration and centrifugation at 10,000×g for 20 min to remove the mycelium and bud spore. CF was sterilized by filtration through a 0.22 μm membrane, adjusted pH to 5.8 and used immediately or stored at −20°C until use.

CF (10 m*l*) was separate into two fractions using a dialysis tube with a molecular cutoff of 12-14 KDa against 500 m*l* of sterile distilled water for 16 hours at 4°C with two changes of the external solution. The internal solution was used as high molecular weight fraction (HMW), and the external solution which was concentrated to the same volume as the internal solution was used as low molecular weight fraction (LMW).

Effect of culture filtrates on protoplasts. Protoplast viability was assayed by exclusion of the dye Evans' Blue[9]. Protoplast preparations were employed and their density was adjusted to 1×10^6/m*l*. Crude CF, HMW and LMW were added to protoplast suspensions at 30 % (v/v) based on SH[10] medium supplemented with 13 % mannitol, the pH of each solution being adjusted to 5.8. The protoplast deaths were recorded after incubation for 3, 6 and 18 hours. The protoplast density was also counted (each time) using a haemacytometer. In this experiment, the decreasing protoplast density coincided with an increase in the percentage of collapsed protoplasts, because the number of collapsed protoplasts could not be counted directly. All treatments were performed in triplicate and were repeated once to give 5 sample readings. In the case of protoplast viability over 100 protoplasts were counted. The percentages of protoplast death and density as a result of each fraction treatment were adjusted by the values obtained from control treatment with SH containing Czapek-Dox, its LMW and HMW fractions, after the same period (typically this viability was 90 ~95 % in each observation).

Results and Discussion

Fig. 1 shows the percentage of protoplasts which reacted to each fraction of *V. albo-atrum* CF.

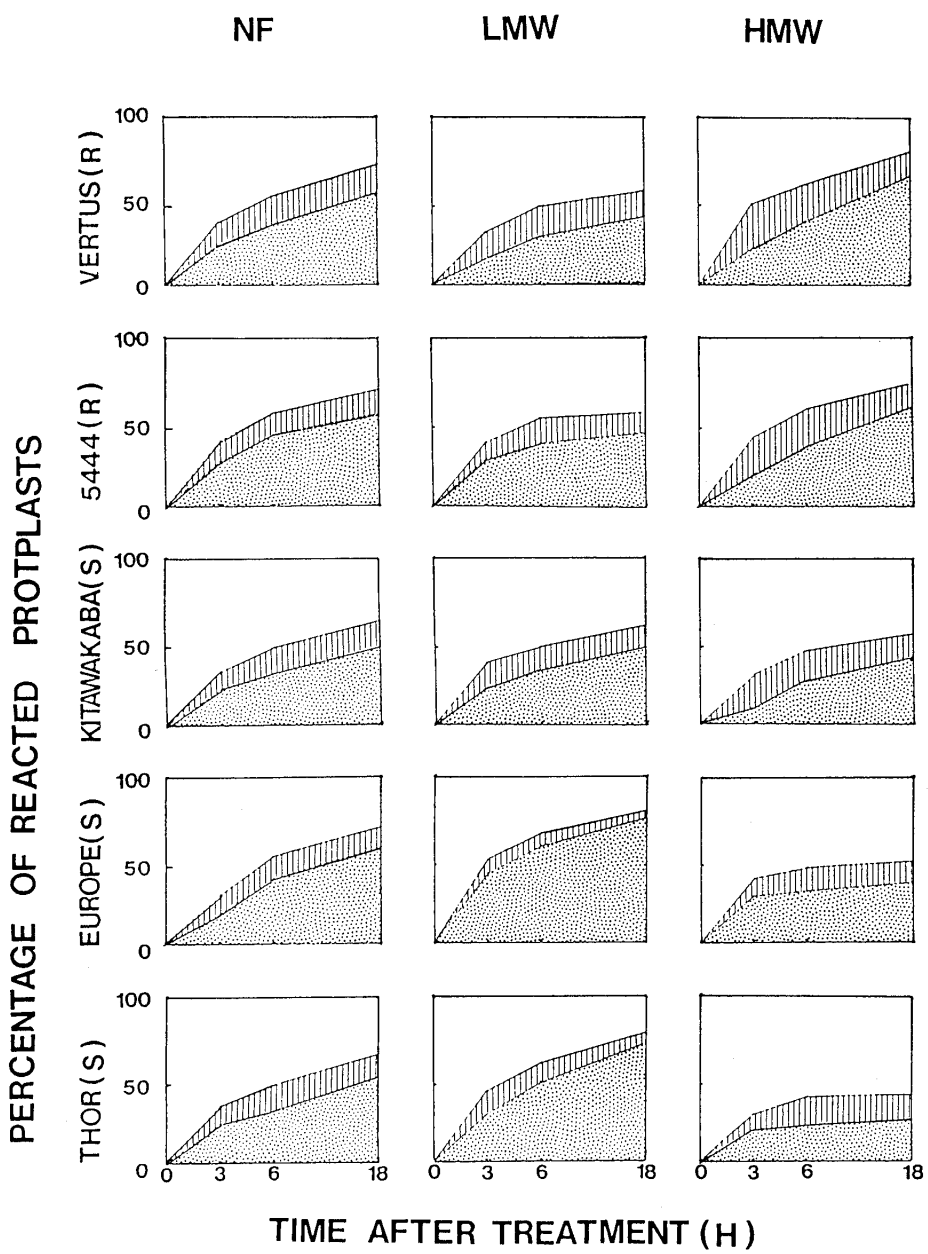

Fig. 1 The time course reactivity of protoplasts obtained from five alfalfa cultivars against the fractions of *Verticillium albo-atrum* culture filtrate (CF). Vertus and 5444 are resistant, and Kitawakaba, Europe and Thor are susceptible to Verticillium wilt. NF: reactions to nonfractionized CF, LMW: reactions to low molecular weight fraction, HMW: reactions to high molecular weight fraction. □: not affected, ▨: death, ▩: collapsed.

In each cultivar, protoplast reactions (protoplast death and density decrease) to non-fractionized CF showed a similar pattern. In this case, there was no significant correlation between *in vivo* resistance and *in vitro* response of the cultivars (**Fig. 2**). This result indicated that the reaction of protoplasts to non-fractionized CF did not reflect their *in vivo* resistant levels. On the contrary, Connell *et al.*[11] showed that the extent of cytotoxity was correlated with both the virulence of the isolates and the resistance of the cultivars in hop protoplast-*V. albo-atrum* interaction. The

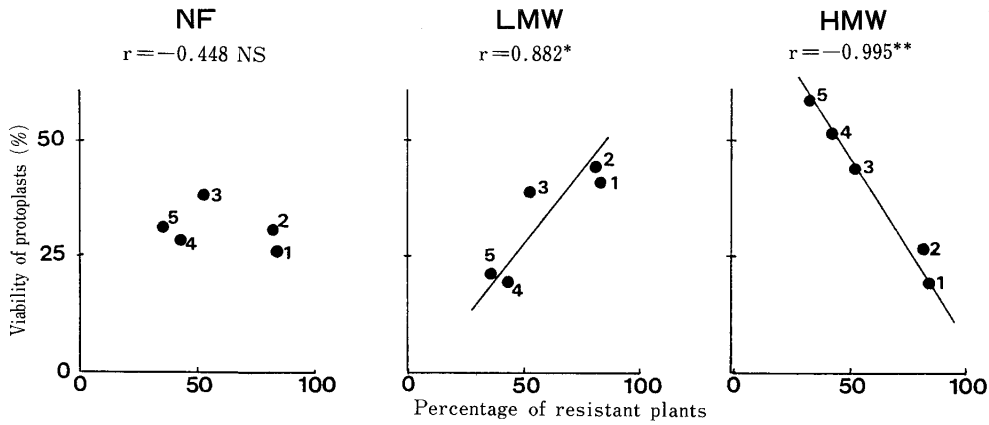

Fig. 2 Relationships between viabillities of alfalfa protoplasts after 18 h incubation in the medium containing *Verticillium albo-atrum* culture filtrate (CF) fractions and percentages of plants resistant to Verticillium wilt. NF: reactions to non-fractionized CF, LMW: reactions to low molecular weight fraction, HMW: reactions to high molecular weight fraction. 1 : Vertus, 2 : 5444, 3 :Kitawakaba, 4 : Europe, 5 : Thor. NS : not significant, *significant (p=0. 05), **significant (p=0. 01).

difference between their result and ours may be due to a different plant species or the CF condition.

The protoplast reaction to LMW CF fraction, on the other hand, exhibited different patterns comparable to *in vivo* resistance of cultivars (**Figs. 1, 2**). This result indicates that the protoplast viability to LMW CF of *V. albo-atrum* may be used for a marker for *in vivo* resistance.

The protoplast reaction to HMW CF was similar to LMW CF but the direction of response was opposite. Protoplast derived from resistant cultivars reacted more quickly and highly than those of susceptible cultivars (**Fig. 1**). There was a good negative correlation ($r=-0.995$, $p=0.01$) between *in vivo* resistance (% of resistant plants) and protoplast viability to HMW CF fraction (**Fig. 2**). Such correlation between the reactivity of protoplasts to hyphal cell-wall components and *in vivo* (horizontal) resistance has also been documented in the cases of potato leaf blight[12,13] and rice blast[14]. Our results indicate that HMW CF may contain some substances whose activity was the same as fungal cell wall components.

From these results the reason the reaction of protoplasts to non-fractionized CF showed no cultivar-specifity seems to be apparent. Namely, since non-fractionized CF contained both LMW cytotoxic components (susceptible cultivar-specific) and HMW cytotoxic components (resistant cultivar-specific), the effects of these components were counteracted and masked. In addition to this explanation, the question is raised as to why the rate of reacted (dead and collapsed) protoplasts to non-fractionized CF is lower than the total rate of protoplast reactions to LMW and HMW CF. We, however, cannot explain this phenomenon.

Finally, these results lead to the conclusion that the percentages of protoplast viability to LMW CF and reacted protoplasts to HMW CF may be a good marker for *in vivo* resistance to Verticillium wilt. If we use the LMW CF of *V. albo-atrum* as the *in vitro* selective agent, the investigation into the effect on protoplast division and calli development of the media including LMW CF can be accomplished.

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《和文要訳》

アルファルファと *Verticllium albo-atrum* の相互作用
III. 菌培養濾液に対するアルファルファプロトプラストの反応

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抵抗性程度の異なるアルファルファ5品種の子葉から単離したプロトプラストのバーティシリウム萎ちょう病菌の培養濾液に対する反応を調べた．培養濾液全画分に対する反応では，死細胞率増加および密度低下を示したが，品種間の差は認められなかった．しかし，低分子画分（<12-14, 000）に対しては抵抗性程度の低い品種ほどプロトプラストの生存率が顕著に低下し，高分子画分（>12-14, 000）に対しては抵抗性程度の高い品種ほどプロトプラストの生存率が顕著に低下した．