Effects of *Verticillium albo-atrum* culture filtrate on somatic embryogenesis in alfalfa

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**ABSTRACT**

Cell suspensions derived from young petioles of alfalfa (*Medicago sativa* L.) were cultured in the presence and absence of a *Verticillium albo-atrum* culture filtrate (20% v/v) for 6 cycles. The frequency of somatic embryogenesis and the growth rate of the suspension cultures were investigated at each cycle. Somatic embryogenesis in the filtrate-treated cultures declined but was still at a relatively high level after 6 subcultures, compared to controls cultures which virtually lost the capacity for embryo formation in the same period. The decline in the embryogenic capacity of filtrate-treated cultures was accompanied by a six-fold increase in the rate of growth of the cultures.

**INTRODUCTION**

In vitro selection has been successfully used to isolate disease-resistant lines in a number of crop species (Wenzel, 1985). The main advantages of selecting in vitro for disease resistance are that: (1) experimental units can be maintained on defined media and in a rigorously controlled environment which facilitates the measurement of slight quantitative difference in polygenically inherited resistant traits; (2) cultured cells can be uniformly exposed to selective agents, thus reducing the incidence of escapes; and (3) culture systems can be maintained in a small space thus replacing large field requirements. However, in order to realize these advantages it is important that the plant materials have a high regeneration capacity. This is particularly true if mutagenic treatments are not used and somaclonal variants arising spontaneously in culture are being selected.

A protocol that has been widely used for the selection of disease resistant lines is to grow callus or suspension culture cells in the presence of a fungal culture filtrate or purified fungal toxin (Sacristan, 1982). The assumptions in this approach are that toxic metabolites in the filtrate produced by the pathogen play a role in pathogenesis and that they can be used to exert a selection pressure for cells that are resistant to the pathogen. Although this method does not assure that plants regenerated from resistant calli or suspension cells will be also resistant to the pathogen, it has been effectively applied to several plant-pathogen systems (Arcioni et al. 1987; Gengenbach et al. 1978; Hartmann et al. 1994; Ling et al. 1985).

In vitro selection systems for selection of *Verticillium albo-atrum* resistant alfalfa are theoretically feasible because highly embryogenic culture systems based on genotypes selected from Rangelander (Heinrichs et al., 1979) and Regen-S (Bingham et al., 1975) have been defined and the toxicity of the fungal culture filtrate to alfalfa plants (Panton, 1967) and tissue culture has been demonstrated (Frame, 1988). However, the effects of filtrate on somatic embryogenesis of alfalfa are not documented.

An interesting phenomenon observed during experiments with embryogenic alfalfa cultures exposed to *Verticillium albo-atrum* culture filtrate is reported herein that suggests that the filtrate has different effects on embryogenic and non-embryogenic cells in culture.

**MATERIALS AND METHODS**

**Plant material**

The alfalfa (*Medicago sativa* L.) genotype used in the present study was A70-34 (a highly embryogenic genotype selected from Rangelander provided by D. C. W. Brown, Agriculture Canada, Ottawa Research Station). This genotype is susceptible to *Verticillium albo-atrum*. The donor plants were maintained in a growth room set to a 16 h light (400-500 μE·m⁻²·s⁻¹) / 8 hour dark photoperiod with 23°C light / 16°C dark temperatures.

**Preparation of the fungal culture filtrate and toxic media**

Potato dextrose agar (PDA) in petri dishes was inoculated with a virulent *Verticillium albo-atrum* isolate of alfalfa obtained from G. Boland, Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada, and incubated at 22°C in the dark. Two weeks later one piece of PDA with fungal mycelium was transferred to liquid B5 medium (Gamborg et al. 1968) without growth regulators. The cultures were kept at 22°C in dim light with continuous agitation (90 rpm), and subcultured by transferring 20 ml of the fungal culture to 30 ml of fresh B5 medium at two-week-intervals. Any flasks showing abnormal or no mycelial growth were discarded. Fungal cultures
that had been subcultured at least twice were filtered first through filter paper, then sterilized with a 0.2 um Nalgene filter unit.

Toxic alfalfa culture were prepared by substituting fungal culture filtrate for B5 in the B5g media (Senaratna et al., 1988). To avoid thermal degradation of toxic compounds in the fungal culture filtrate, it was added after the media had been sterilized.

**Callus and suspension culture**

Petioles from young leaves were used to initiate callus on solidified B5h media (Atanassov and Brown, 1984). In three to four weeks, petioles which had callused well were transferred to liquid B5f media to initiate the cell suspension cultures. One week later the suspension cultures were used to inoculate B5f medium containing 20% (v/v) fungal culture filtrate and a control flask containing 20% (v/v) B5 to begin the first cycle. The cell suspensions were subcultured at 12 day intervals in the same media for a total of six cycles.

**Somatic embryogenesis**

At the end of each cycle, equal volumes of the filtrate-treated and the control cultures were filtered through separate sterile nylon screens (224 um mesh) to remove the single cells and collect the cell clusters. The cells collected by the screens were transferred to fine nylon screens (63 um mesh) on B12y (Atanassov and Brown, 1984) solid media in petri dishes. Each petri dish was weighed before and after transfer of the cells and cell fresh weight was obtained by subtraction. The cells were incubated at 25°C with 16-hour day length and the embryos that had formed after 3-4 weeks were counted.

**RESULTS AND DISCUSSION**

The effects of Verticillium albo-atrum culture filtrate on somatic embryogenesis in A70-34 cultures are shown in Fig. 1. The embryo production frequency of the filtrate-treated cultures increased from the first to the second cycle but declined during subsequent cycles. However, in spite of the decrease in the later cycles the embryo production frequencies remained relatively high throughout the culture period; over 500 embryos/g cells were produced during the 6th cycle (11 weeks). In contrast, embryo formation from control cultures declined from over 2,000 embryos/g cells in the first cycle to 250 embryos/g cells in the second cycle and the cultures virtually lost the capacity for embryo formation by the sixth cycle.

![Figure 1. Embryo production by alfalfa cultures grown in six cycles (C1-C6) in the presence (solid bars) or absence (cross hatched bars) of 20% (v/v) Verticillium albo-atrum culture filtrate after plating onto B12y medium; n=4.](image)

During the first four subcultures the growth rate of control cultures remained constant at 11% initial packed cell volume/day (IPCV) but the growth rate of filtrate-treated cultures increased progressively from 1% IPCV/day during the first cycle to 6% IPCV/day during the fourth (Fig. 2).

The decline in the growth rate of control cultures is similar to previous reports with other species (Barba and Nickell, 1969; Lustinec and Horak, 1970; Kev Kishor and Reddy, 1987). As postulated by Fridborg and Eriksson, (1975) and Negriuti and Jacobs (1978) this could be because of an altered hormonal balance within the cells or a change in the sensitivity of the cells to exogenous growth substances or may be due to genetic changes (Sheridan, 1975). Alfalfa suspension cultures derived from embryogenic lines contain a mixture of cell types including:

1) highly vaculated, undifferentiated cells
2) pro-embryogenic clusters containing small, cytoplastic cells in their centers and enlarged cells at the margin, and
3) dense callus clusters (B. Mersey, personal communication).

The results of the present study suggest that the Verticillium culture filtrate inhibits the growth of non-embryogenic cells but does not affect the embryogenic cells in A70-34 cultures. In fact, when the suspension cultures are plated onto solid medium containing filtrate the undifferentiated cells that normally develop into light brown callus on control media turn black and fail to develop. However, the proembryos are not affected by the filtrate and continue to develop into green embryos (data not shown). We propose that as a consequence of this differential sensitivity to the filtrate among the different types of cells the embryogenic capacity of the filtrate-treated cultures is prolonged. In this study the acquisition of tolerance to the Verticillium filtrate by the alfalfa cells results in a six-fold increase in their growth rate and a four-fold reduction of the embryogenic capacity.

The results can be explained if it is assumed that the undifferentiated cells have a higher growth rate than the proembryogenic clusters. In an unchallenged culture the undifferentiated cells would become predominant and the embryogenic capacity would be lost. However, in the filtrate-challenged cultures the high sensitivity of the undifferentiated cells and the relative insensitivity of the proembryos to the fungal toxin(s) would have the effect of maintaining the embryogenic capacity of the culture.