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Direct Infection of Embryogenic Tissue Cultures of Haploid *Brassica napus* with Resting Spores of *Plasmodiophora brassicae*

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Summary. A direct infection of cultured tissue with resting spores of the obligate endoparasite *Plasmodiophora brassicae* has been made possible by using stem embryo cultures of haploid rape. The fungus develops and completes its life-cycle in the cultured cells. The results are discussed in connection with the possibility of using this system to select for resistance to the pathogen.

Key words: *Plasmodiophora brassicae* – Resistance in tissue cultures – Infection of cultured tissue

Introduction

Plasmodiophora brassicae, the causal agent of club root disease of the Cruciferae, is an obligate fungal parasite which remains in the host cells during its entire life cycle. The callus culture of infected tissue allows the establishment of dual host-parasite cultures in which the fungus proceeds through all stages of its life history (Ingram 1969 a; Williams et al. 1969; Tommerup and Ingram 1971; unpublished own results). In these dual cultures, the formed resting spores probably germinate in situ and, in this way, a new cycle begins. Attempts to directly infect healthy callus cells with isolated plasmodia have failed (Dekhuijzen 1975).

In this paper we report our attempts on direct infection of healthy tissue cultures with contaminant-free resting spores suspensions. Successful infections occurred in stem embryo cultures of haploid *Brassica napus*. These cultures consist of hypocotyl-like structures which develop embryoids from single cells of their epidermis (Thomas et al. 1976). The idea for using such a system is based on the fact that in natural conditions the parasite is able to infect hypocotyls.

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Material and Methods

Stem embryo cultures were obtained from regenerating plantlets of protoplast cultures of haploid androgenetic rape, *Brassica napus*, spring line 24/72 (Thomas and Wenzel 1975; Thomas et al. 1976; Wenzel et al. 1977). They were propagated on the basic medium of Murashige and Skoog (1962) without hormones, with 1% sucrose and 0.8% agar, at 25°C and under a light intensity of 1500 lux. For infection experiments the cultures were transferred to liquid medium of the same composition but without agar.

In parallel experiments with cell cultures of haploid rape, callus colonies of single-cell origin which developed in suspension cultures were used. The cell suspensions were obtained from callus which had been placed on a giratory shaker (120 rpm) in the liquid medium of Murashige and Skoog (1962) with 0.1-0.2 mg/l 2,4-D and 10 mg/l kinetin.

The strain of *Plasmodiophora brassicae* utilized for inoculations was isolated from Chinese cabbage in the locality of Borken/Westfalen. It induced 100% disease in rape plants. Its propagation was carried out on Chinese cabbage.

To obtain a contaminant-free resting spores suspension, clubbed roots of 6-8 week old plants were surface-sterilized in 1% $HgCl_2$ (10 min), 70% ethanol (1 min) and thoroughly washed with sterile water. They were then homogenized in sterile water and the homogenized material was filtered through 80μ sieves to remove debris. The spore suspension was washed by means of repeated centrifugations. The sediment of the last centrifugation was suspended in culture medium to a concentration of approximately 10^8 spores/ml, and this suspension tested for sterility.

For the microscopical analysis, the tissues were fixed in ethanol – acetic acid 3:1, stained in 2% acetic orcein – 1N HCl 9:1 and squashed in acetocarmin.

Results

Two hundred inocula from stem embryo cultures which had been precultivated for two weeks on liquid nutrient medium were transferred to the same medium containing resting spores of *P. brassicae* at a final concentration of 6.5×10^7 spores/ml. The same procedure was applied to 200 small cell colonies from suspension cultures which in the liquid medium had developed profuse roots and root hairs.

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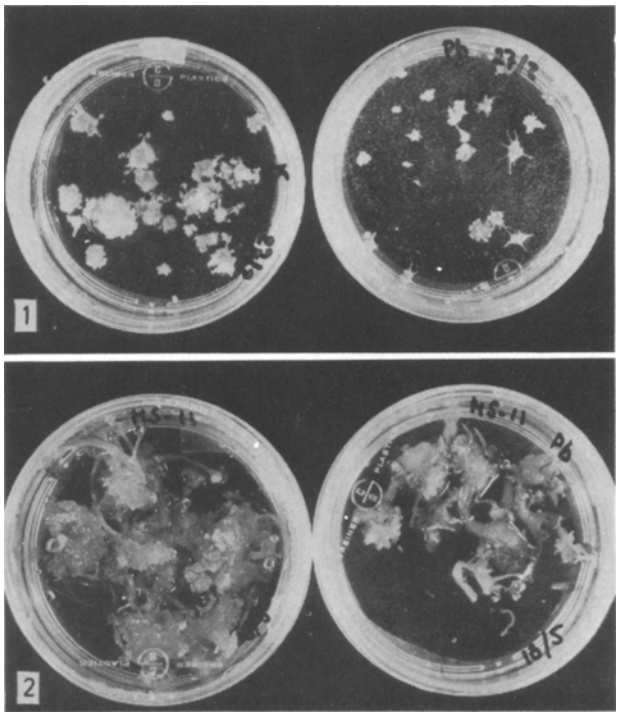

Fig. 1. Control (left) and with resting spores of *P. brassicae* treated callus colonies (right) from suspension cultures (11 days on medium containing 10^7 spores/ml)

Fig. 2. Control (left) and with resting spores of *P. brassicae* treated stem embryo cultures (right) on liquid nutrient medium (20 days on medium containing 6.5×10^6 spores/ml)

Five to ten days after the transfer to the medium with spores, a marked growth inhibition of 50-70% in respect to the controls could be observed in both cultures (Fig. 1-2). In the stem embryo cultures, this inhibition was accompanied by an inhibition of the chlorophyll synthesis, the cultures becoming at first white, then brownish.

After 20 days of culture on infected medium, 80% of the tissue mass was necrotized, whereas living parts of the embryogenic tissue showed a tendency to form swollen structures and unorganized callus. The tissues were then washed with nutrient medium to remove adhering spores and transferred onto agar medium for further culture. Again, on this medium, 70-80% of the treated culture became white and degenerated. Occasionally, in stem embryo cultures, green fast-growing tissue regenerated from a point of a degenerating culture.

Microscopical analyses carried out 2-7 days after the start of the treatment with spores showed that spores germinated and penetrated into the cells of both types of cultures. However, whereas formation of plasmodia and further development of the fungus could not be observed in callus cultures, beginning 7 days after the inoculation, a high proportion of cells of the stem embryo cultures contained plasmodia and different phases of the life cycle of the parasite (Fig. 3-5). In these cultures plasmodial phases and sporulation could be observed up to eight weeks after infection (Fig. 6-8). These observations indicate that the parasite can infect, complete its life cycle and reinitiate new cycles in the in vitro culture conditions.

Discussion

The reported results introduce the possibility of using in vitro systems, not only for the study of infection and development of *P. brassicae* in controlled experimental conditions, but also to induce and select embryos or cell lines resistant to the parasite. Mutagenic treatment can now be combined with the selection process. Using the advantages of stem embryo cultures, e.g., large numbers of secondary embryoids from single haploid cells, it was possible to induce mutations and to isolate homozygous mutant plants (Hoffmann 1978).

A previous condition for the use of in vitro systems to induce and select resistant lines to an obligate parasite is the capacity of the parasite to infect directly the cultures. In the case of *P. brassicae*, it does not suffice that the spores germinate and penetrate in the host cells because the first phase of its life cycle, the 'root hair phase', has also been reported in nonsusceptible hosts (Colhoun 1958). The parasite must be able to complete its life cycle up to the formation of resting spores. We demonstrated that this occurred in the assayed system of stem embryo cultures.

If it were possible to infect directly single cells or unorganized callus, with subsequent development of the fungus in these cells, a screening system for resistance could be based on the differential hormonal requirements of *P. brassica*-infected and non-infected cells, a fact which was observed by Ingram (1969 b) and Williams et al. (1969) and subsequently confirmed by our own results (unpublished).

The observation in stem embryo cultures that infected parts of the tissue lose their capacity to synthesize chlorophyll and to undergo embryogenesis could serve as a criterion for a screening system for resistance: regenerated embryoids from degenerating browning regions of the tissue could be selected as being possibly resistant.

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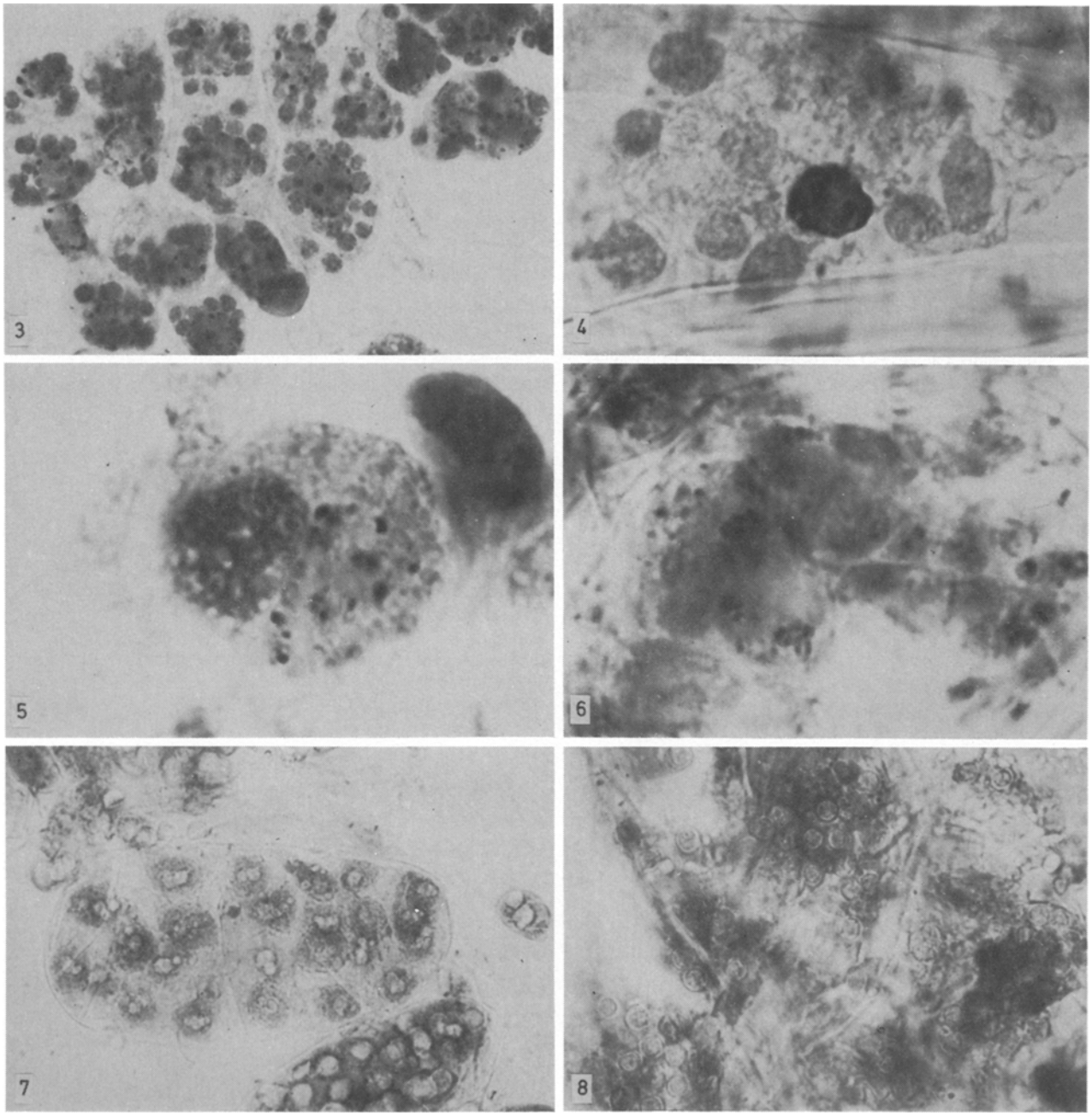

Figs. 3-5. *P. brassicae*-infected cells of stem embryo cultures after 7 days culture on medium with spores. **Fig. 3.** Heavy infection of culture cells with germinated spores; nuclei of the host cells with heterochromatic chromocenters (1200 X) **Fig. 4.** Cell with several plasmodia; host cell nucleus dark stained (1800 X). **Fig. 5.** Cell with one plasmodium besides the nucleus (2400 X)

Fig. 6. Plasmodium in cleavage and divisions in a degenerating cell, 4 weeks after the start of the infection (2400 X)

Figs. 7-8. Infected cells five weeks after the transfer from medium with spores to agar medium. In Fig. 8, formation of resting spores (1200 X)

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