Superoxide Anion Generation: A Response of Potato Leaves to Infection with *Phytophthora infestans*

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ABSTRACT

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In potato leaves inoculated by a zoospore suspension of *Phytophthora infestans*, O_2^- generation appeared at two stages, before and after fungus penetration. The former reaction was only temporal in leaves that were inoculated with either the compatible or incompatible races of *P. infestans*. This reaction was also activated by zoospore germination fluid. The latter reaction, however, was characteristic of the incompatible, but not the compatible interactions, with respect to the occurrence of a hypersensitive cell reaction following fungus penetration. An O_2^- generating reaction before fungus penetration was also found to be activated in some nonhost plant leaves that were either inoculated with the fungus or treated with the germination fluid. However, a few penetrating fungi in these nonhost plants resulted in no detection of enhanced O_2^- generation after fungus penetration. These results suggest that intact leaves may recognize some substance released from germinating spores that activates an O_2^- generating reaction, and that further activation of O_2^- generating reaction may occur in potato leaf tissues in close association with a hypersensitive reaction elicited by penetration by incompatible races of *P. infestans*.

It has been well documented that in aged potato tuber slices a hypersensitive reaction occurs characterized by rapid cell death (17,18,25) and subsequent production of phytoalexin (5,19,23,24,27) against invasion by incompatible, but not compatible, races of *Phytophthora infestans* (Mont.) de Bary. Little potential for hypersensitive reaction has been observed in fresh potato tuber slices (14,20). During aging, after slicing, cell activation is necessary for the cell to react hypersensitively to infection with incompatible races of *P. infestans* (14,20).

Recently, a novel biochemical reaction, O_2^- generation, was found to be activated in aged, but not fresh potato tuber slices after fungus penetration of potato cells by incompatible, but not compatible, races of *P. infestans* (6). The O_2^- generation was suggested to occur in close association with the cell activation after slicing and the occurrence of a hypersensitive cell death (6). The O_2^- generation was found to be mediated by a novel NADPH (β-nicotinamide adenine dinucleotide phosphate) oxidase, presumably in host plasma membrane (7,8).

Intact potato leaves are also known for hypersensitive reaction against invasion by incompatible, but not compatible, races of *P. infestans*. In this case, it has been also demonstrated that cell activation responsible for the occurrence of a hypersensitive reaction may be a prerequisite before fungus penetration (15,16). There is very little information on the biochemical changes that occur in potato leaves infected with *P. infestans*. It is of interest to determine if leaf tissue responds to fungal infection by O_2^- generation in a manner similar to tuber slices.

In the present paper, aspects of O_2^- generation in intact potato leaves inoculated by compatible and incompatible races of *P. infestans* are described. As references, O_2^- generation in other nonhost leaves was also determined. A brief report of this study has been published elsewhere (3).

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MATERIALS AND METHODS

Plants. Host plants used were potato cultivar Rishiri carrying R_1 resistance gene to *P. infestans* (an interspecific hybrid between *Solanum tuberosum* L. and *S. demissum* L.) and Irish Cobbler without resistance genes (*S. tuberosum*). Plants were grown in pots (25 cm diameter, 19.5 cm depth) under field conditions in the spring. The seventh compound leaf from the apical bud of each plant was detached 6–7 wk after planting, and four leaflets of this leaf, except the top leaflet, were used. Nonhost plants used were tobacco, bean, soybean, and cowpea. Tobacco, *Nicotiana tabacum* L. 'Samsun NN', plants were grown in pots (12 cm diameter, 8.5 cm depth) for 3 mo in a greenhouse and leaves about 10 cm long were used. Bean (*Phaseolus vulgaris* L. 'Americana'), soybean (*Glycine max* Merr. 'Suzushigeshiratoriedamame') and cowpea (*Vigna sinensis* Kurodane sanjaku) were grown in pots (8 cm diameter, 6.5 cm depth) in a greenhouse, and their primary leaves, those that form before the first true leaves, were used. In all experiments, leaf disks, 10 mm in diameter, were cut from the leaves using a cork borer and washed with distilled water to remove the contents of any destroyed cells in cut area. Five leaf disks were used for each assay.

Fungus and inoculation. The *P. infestans* incompatible race 0 and compatible race 1.2.3.4 were used. A zoospore suspension for use as inoculum was prepared for each race as previously reported (4), and adjusted to be a concentration of 5×10^5 spores per milliliter. Inoculation was done by applying 50 μl of the zoospore suspension on the upper surface of each leaf disk covered with lens paper (Kodak Co.), which was used for quantitative and uniform inoculation. Inoculated leaf disks were incubated on wetted filter paper in a moist chamber at 20 C in the dark.

Preparation of germination fluid. A zoospore suspension (5×10^5 spores per milliliter containing 10^{-3} M $CaCl_2$ was shaken at 100 strokes per minute at 20 C for 5 hr to promote synchronous zoospore germination as described previously (11). The suspension was then centrifuged at 20,000 *g* for 30 min and the germination fluid collected by filtering the supernatant through a membrane filter (TM-4, pore size 0.2 μ, Toyo Filter Paper Co.).

Treatment of leaves with germination fluid. Fifty microliters of

the germination fluid was applied on the upper surface of each leaf disk covered with lens paper, similar to incubation.

Assay of O_2^- generation. Determination of O_2^- generated from leaf disks was done by assaying spectrophotometrically the reduction of extracellular cytochrome c as described previously (6). At intervals after inoculation, a set of five leaf disks was immersed in 3 ml of 0.05 M K-phosphate buffer (pH 7.8) containing 20 μm of cytochrome c (Type VI, from horse heart, Sigma Chemical Co.) and 0.1 mM ethylenediaminetetraacetic acid (EDTA). In each assay, about 3 ml of the reaction solution was pipetted out, the optical density at 550 nm was measured by a double-beam spectrophotometer (Hitach Co. Ltd., type 200-20), and the solution was then returned to the disks. The measurement was done every 2 min for 10 min. Increase in OD_{550} was almost linear for at least 10 min after initiation of the reaction. Cytochrome c reducing activity of the disks was expressed as μmoles per 5 disks per 10 min by using an extinction coefficient of 21.1 (reduced-oxidized) (26). The reduction of cytochrome c by O_2^- was confirmed by measuring the reduction in the presence of 100 μg/ml of superoxide dismutase (SOD) (Sigma Chemical Co.).

Microscopic observations. Inoculated leaf tissues were periodically immersed in a boiled mixture of lactic acid, phenol, and ethanol (1:1:2, v/v/v) for 1.5 min and further incubated in this mixture at room temperature overnight. Discolored leaf tissues were stained with 0.1% Calcofluor White M2R New (American Cyanide Co., kindly given by Dr. Komoto and Dr. Otani, Tottori University) according to the method of Rohringer et al (22). The leaf specimens were then mounted in glycerol containing a trace of the mixture of lactic acid and phenol (1:1, v/v). The mounted specimens were then examined with a fluorescence microscope (Olympus, BHA-LB) under incident light from an HBO 100 ultra-violet lamp (DM 400). Excitation filter UG1 was used in combination with barrier filter L-420.

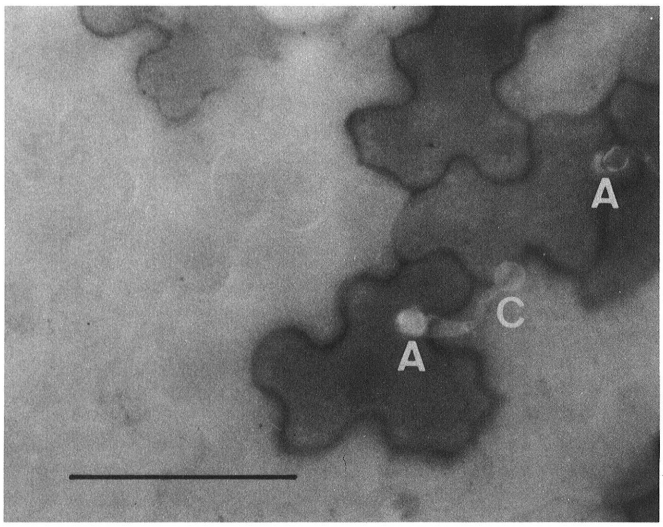

Fig. 1. Fluorescence microscopic observations of the potato leaf surface inoculated by the *Phytophthora infestans* incompatible race 0. Leaf samples 5 hr after inoculation were stained by Calcofluor White M2R New. A: Appressoria. C: Cystospore. Scale bar represents 100 μm.

RESULTS

Time course of appressorium formation, fungus penetration, and hypersensitive cell response. Cystospores, germinating hyphae, appressorium, and intercellular hyphae of fungi were observed with strong whitish-blue fluorescence under the present experimental conditions (Fig. 1). Host cells also showed such fluorescence. It was found that most of the epidermal cells that were penetrated by the incompatible, but not by the compatible race of *P. infestans*, became dark blue (or less fluorescent) with time after fungus penetration (Fig. 1). The appearance of less fluorescence in cells penetrated by the fungus was interpreted as a sign of incompatible response. This was based on the evidence that the number of such cells was almost consistent with that of browned cells observed by the light microscope at 24 hr after inoculation. Also, few cells penetrated by the compatible race of *P. infestans* exhibited the reduced fluorescence.

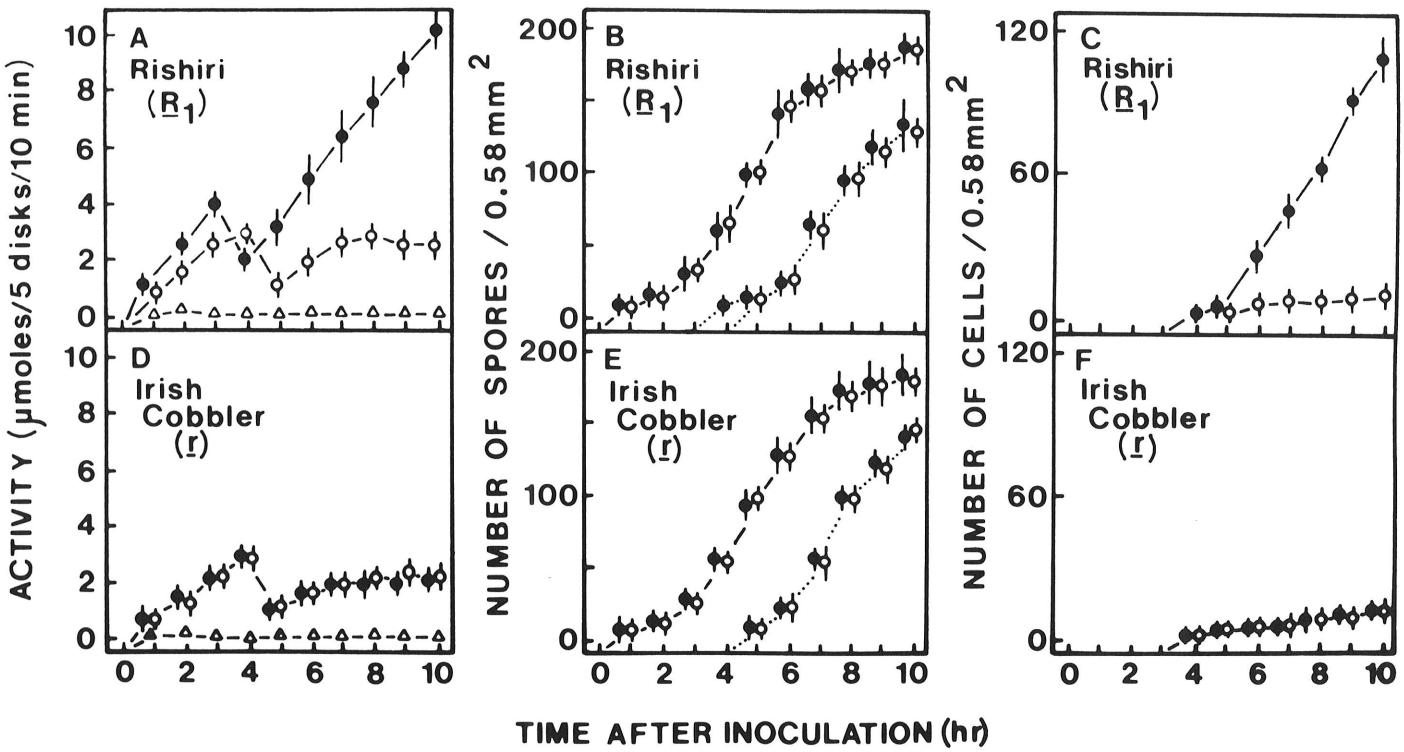

Fig. 2. Time course change in O_2^- generating activity of potato leaf tissues (cv. Rishiri, R_1, and cv. Irish Cobbler, *r*) after inoculation by the *Phytophthora infestans* incompatible race 0 and the compatible race 1.2.3.4 in relation to appressorium formation and invasion of the fungus and hypersensitive response of host cells. **A and D**: O_2^- generating activity determined by cytochrome c reducing activity. **B and E**: Germinated cystospores forming appressoria (---) and intercellular hyphae (···). **C and F**: hypersensitively reacted cells penetrated by fungi. •: *P. infestans* incompatible race 0, o: *P. infestans* compatible race 1.2.3.4, Δ: uninoculated. Each value represents an average of nine determinations ± S.D. in three separate experiments.

In leaves inoculated by incompatible and compatible races, appressorium formation started 1 hr after inoculation, increased with a sigmoidal curve, and reached a maximum rate at about 8 hr after inoculation (Fig. 2B and E). The fungus penetration started from 4 hr after inoculation and increased almost linearly until the last observed time, 10 hr after inoculation (Fig. 2). The incompatible cell response was observed from 5 hr after inoculation and the number of hypersensitive reacting cells increased with increase in number of penetrating fungi in epidermal cells inoculated with the incompatible, but not compatible, race (Fig. 2C and F).

O_2^- generation in potato leaves inoculated with fungus. Potato leaf disks (cv. Rishiri, R_1) inoculated with the *P. infestans* incompatible race 0 showed a potential for reducing extracellular cytochrome c (Fig. 2A). The reducing activity appeared before the fungus began penetration into host cells (about 4 hr after inoculation) and greatly increased with times after fungus penetration. The latter activation seemed to occur in association with an increase in the number of fungus penetrated cells, which reacted hypersensitively (Fig. 2B and C). In the leaf disks inoculated with *P. infestans* compatible race 1.2.3.4, the initial increase in cytochrome c reducing activity before fungus penetration was also shown to be similar to that of leaf disks inoculated by the incompatible race 0 (Fig. 2A). However, the cytochrome c reducing activity increased little with increase in the number of cells penetrated by the fungus (Fig. 2A and B). These penetrated cells did not show a hypersensitive reaction (Fig. 2C). There was not any difference in the rates of fungal penetration into host cells between the compatible and incompatible races (Fig. 2B). Irish Cobbler (*r*) leaf disks compatible with both *P. infestans* races used showed quite similar patterns for the activation of cytochrome c reducing activity following inoculation with these races as compared with Rishiri (R_1) disks inoculated by the *P. infestans* compatible race 1.2.3.4 (Fig. 2D). In uninoculated leaf disks of both Rishiri (R_1) and Irish Cobbler (*r*), little activity was evident to reduce extracellular cytochrome c (Fig. 2A and D). These results indicated that the reduction of cytochrome c may be caused by some reducing agents generated from inoculated leaves.

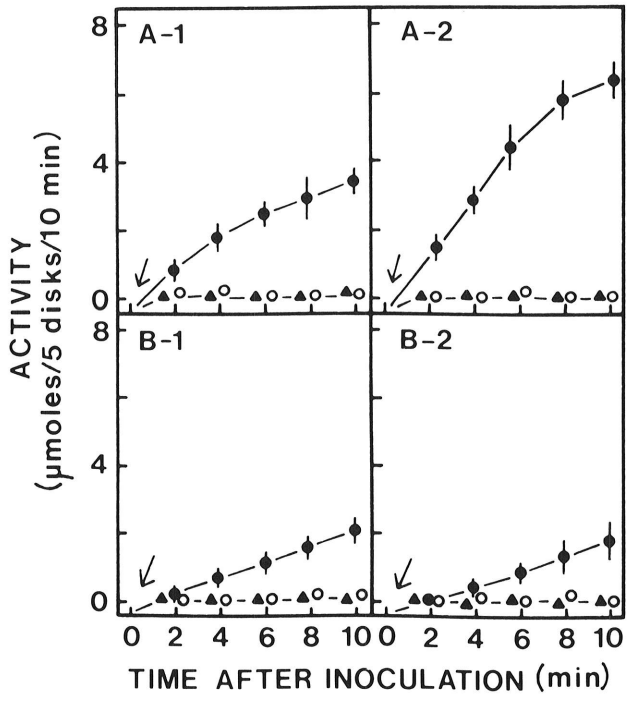

Fig. 3. Extracellular cytochrome c reducing activity of potato leaf tissues (cv. Rishiri [R_1]: **A**; cv. Irish Cobbler [*r*]: **B**) inoculated by the *Phytophthora infestans* incompatible race 0 in the presence (o) or absence (•) of superoxide dismutase (SOD)(100 μg/ml). Leaf disks 3 hr (A-1 and B-1) and 7 hr (A-2 and B-2) after inoculation were used for the assay. ▲: Uninoculated tissue in the absence of SOD. Arrow shows the time of SOD addition. Each value represents the average of nine determinations ± S.D. in three separate experiments.

The reducing activities of inoculated leaves at stages before fungus penetration and after fungus penetration by the *P. infestans* incompatible race 0 disappeared rapidly when the disks were removed from the solution containing cytochrome c (data not shown). This indicated that the reducing agent was closely connected with that of the inoculated tissues. In the reaction mixture that contained the inoculated disks with the cytochrome c reducing activity, the cytochrome c reduction was strongly inhibited by SOD (Fig. 3). Both activities appeared before and after fungus penetration and were very low in the presence of SOD (Fig. 3). Heat-denatured SOD and bovine serum albumin used instead of SOD showed no significant inhibition of the cytochrome c reducing activity. These results suggest that the cytochrome c reducing activity of inoculated leaf disks may be due to O_2^- generated from the disks. The activation of O_2^- generation before fungus penetration may be race-nonspecific, whereas that after fungus penetration may be either characteristic of incompatible interaction or the occurrence of a hypersensitive cell reaction.

O_2^- generation in potato leaves treated with germination fluid. Potato leaf disks from Rishiri (R_1) and Irish Cobbler (*r*) treated with germination fluid from the *P. infestans* race 0 temporally and race-nonspecifically revealed a cytochrome c reducing activity within 4 hr after treatment (Fig. 4A and B). After treatment, the activity increased and reached the maximum 3 hr after treatment and disappeared 4 hr after treatment. Taking into consideration these results, the initial activation of O_2^- generation in inoculated leaves was thought to occur in the leaf tissues as a result of triggering substances released from the germination spores on the leaf surface before fungus penetration.

O_2^- generation in some nonhost leaves. As shown above, it was determined that O_2^- generation by potato leaves was specific in host-*P. infestans* interaction. In nonhost plant leaves (bean, soybean, cowpea, and tobacco) inoculated with zoospores of *P. infestans* race 0, germination of the cystospores took place. However, the rate of germination appeared to be lower than that on potato leaves (Fig. 5). Appressoria formation occurred at a very low rate in these nonhost plant leaves, and few cells penetrated by the fungus were observed. Thus, a hypersensitive cell response to

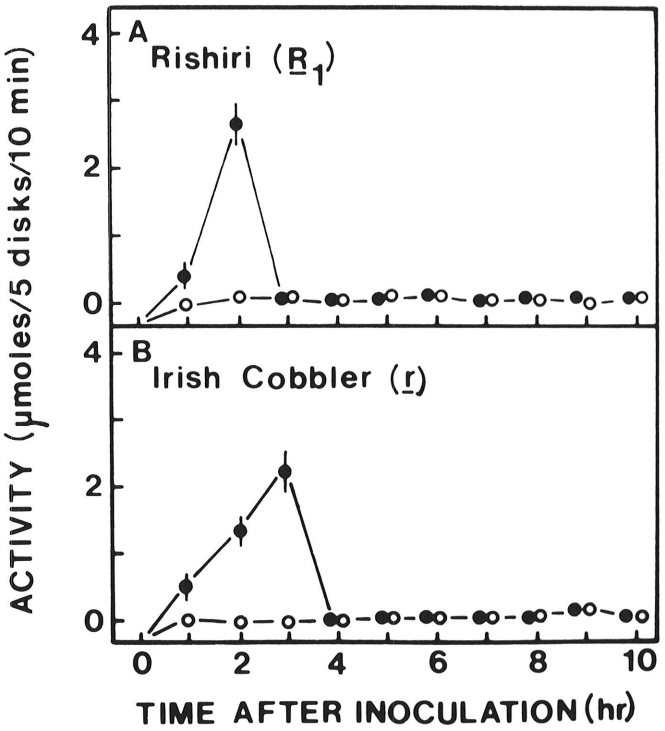

Fig. 4. Elicitation of O_2^- generating activity in potato leaf tissues by germination fluid of the *Phytophthora infestans* incompatible race 0. •: Germination fluid containing 10^{-3} M $CaCl_2$, o: 10^{-3} M $CaCl_2$ solution. Each value represents the average of nine determinations ± S.D. in three separate experiments.

fungus penetration was hardly observed. In these nonhost leaves inoculated by *P. infestans* race 0, the initial O_2^- generation similar to that observed in potato leaves (Fig. 2A and D) was detectable within 4 hr after inoculation (Fig. 5). However, O_2^- generating activity that appeared from 4 hr after inoculation (Fig. 5) was as low as that which appeared in potato leaves inoculated by the *P. infestans* compatible race 1.2.3.4 (Fig. 2A and D). The germination fluid of *P. infestans* (race 0) also caused an activation of O_2^- generation in all nonhost plant leaves used (Fig. 6) similar to that shown in Figure 4 for potato plant leaves.

DISCUSSION

In the present experiments, it may be concluded that potato leaves generate O_2^- as a biochemical response at two stages of infection with *P. infestans*. Although the present method to prove the O_2^- generation was indirect, SOD-sensitive cytochrome c reduction has been accepted to be an evidence demonstrating O_2^- generation in biological systems (1,6,7,9). The O_2^- has been known to be rapidly transformed to H_2O_2, ·OH, or LOOH in biological systems (2,13,21). Therefore, the quantity of extracellular cytochrome c reduction in leaf tissues is seemingly in part the consequence of O_2^- generated in the tissues.

The appearance of two phases of O_2^- generation in potato leaf tissues inoculated by *P. infestans* is interesting in consideration of the host-parasite interaction. The transient activation of O_2^- generation before fungus penetration was evidently race-cultivar nonspecific as well as fungus-host nonspecific. The activation by the germination fluid suggested that some substances released from the germinating cystospores may have nonspecifically caused a physiological change in plants. This means that intact plant leaves may carry a potential to recognize substances produced by the infecting fungus that could shift some cell metabolism before fungus penetration. Because O_2^- and its derivatives can react with many biological compounds (13), it is assumed that the O_2^- generation that occurs before fungus penetration may possibly be involved in some host defense mechanism. Epidermal cells of potato leaves have been demonstrated to be in a condition that lacks the potential to react hypersensitively against incompatible races of *P. infestans*, but that cells can be activated to hypersensitively react between fungal germination on the leaves and fungal penetration (15). Therefore, the initial O_2^- generation activated by both inoculation and treatment with germination fluid may possibly be involved in the activation of the epidermal cells that is responsible for a hypersensitive reaction. It is interesting to consider that sliced potato tuber tissues, which also activate the hypersensitive reactivity during aging (14), activated a transient O_2^- generation immediately after slicing (*unpublished data*).

Secondly, a great activation of O_2^- generation in incompatible interaction of potato leaves revealed that leaves also had acquired a potential to respond to the invading fungus comparable to that of aged potato tuber slices. The aged potato tuber slices with a hypersensitive potential have been shown to generate O_2^-, dependent on NADPH oxidation, in cells penetrated by incompatible, but not compatible, races of *P. infestans* (6). O_2^- generation in this infection stage was also closely associated with the occurrence of a hypersensitive cell death as one of the typical reactions that appeared in incompatible interaction (6). Thus, in

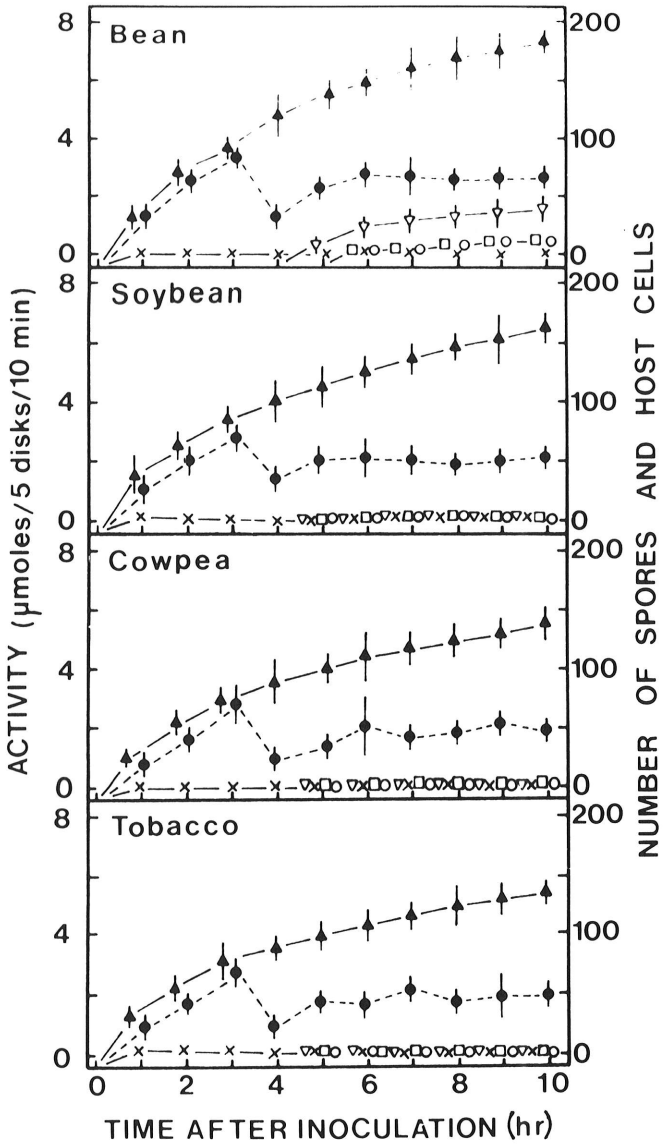

Fig. 5. Time course change in O_2^- generating activity (●) of nonhost plant leaf tissues (bean, soybean, cowpea, and tobacco) after inoculation with the *Phytophthora infestans* incompatible race 0 in relation to germinated cystospores (▲), cystospores forming appressoria (▽), intercellular hyphae (□), and host cell showing hypersensitive response (o). x: O_2^- generating activity in uninoculated leaf tissues. Each value represents the average of nine determinations ± S.D. in three separate experiments.

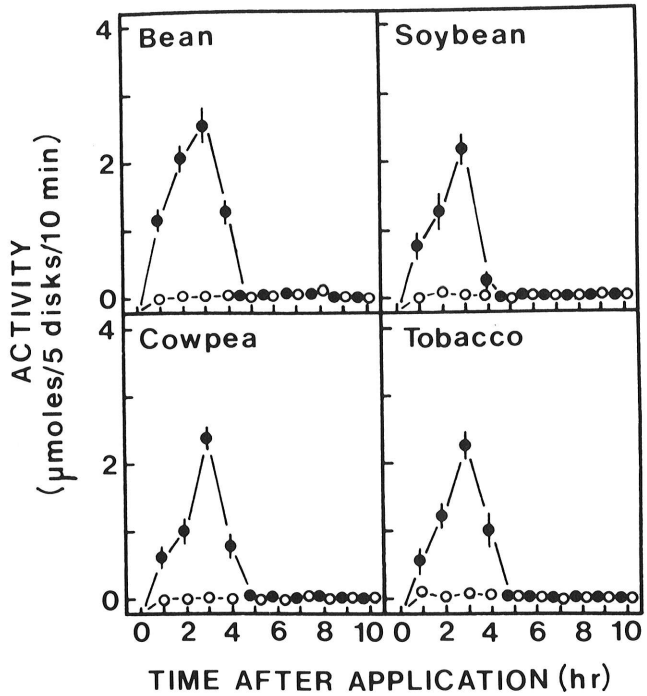

Fig. 6. Time course change in O_2^- generating activity of nonhost leaf tissues following treatment with germination fluid of the *Phytophthora infestans* incompatible race 0. ●: Germination fluid containing 10^{-3} M $CaCl_2$, o: 10^{-3} M $CaCl_2$ solution. Each value represents the average of nine determinations ± S.D. in three separate experiments.

aged potato tuber slices, O_2^- generation was interpreted as a biochemical reaction, responsible for triggering a hypersensitive cell reaction (6–8). Therefore, the similarity of the O_2^- generating reaction that occurred in leaves to that in aged potato tuber slices suggests that the epidermal cell of leaves also reacts to infection with incompatible races of *P. infestans* in a similar fashion to cells in aged potato tuber slices.

The outbreak of O_2^- generation in aged potato tuber slices was assumed to be inhibited in a race-cultivar specific manner by a hypersensitivity-inhibiting factor (HIF) or suppressor (water soluble β-glucans) (10,12) from compatible races of *P. infestans* (7,8). Less activation of O_2^- generation in leaf tissues penetrated by compatible races of *P. infestans* may also be due to suppression by the HIF.

In some nonhost plant leaves used, the initial O_2^- generation observed by inoculation with an incompatible race of *P. infestans* or treatment with its germination fluid was also similar to that observed in potato leaves. These results suggest that the cell response associated with O_2^- generation to *P. infestans* or germination fluid may possibly be common in various plant leaves.

The low activation of O_2^- generating reaction in these nonhost leaves after a 4-hr inoculation with *P. infestans* may be due to few host cells being penetrated by the fungus. It has been previously found that O_2^- generating reactions in potato leaves, as well as other plant leaves (tobacco, bean, cowpea, etc.), were activated by a glycoalkaloid, digitonin (9), and that the reaction was dependent on a NADPH oxidase. Therefore, O_2^- generation in plant leaves that are infected by *P. infestans* may be due to the activation of O_2^- generating NADPH oxidase. To determine the significance of O_2^- generation in plant leaves and its characteristics in more detail further experimentations are required.

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