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BARD

FINAL REPORT

PROJECT No. I-31-79

**Characterization and Mode of Action of
Phytotoxins Produced by *Alternaria dauci*
and Other *Alternaria* spp. and Their
Potential Use in Selection for Disease
Resistant Crop.**

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Date April 1983

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P.O. Box 6
Bet Dagan, ISRAEL

BARD Project No. I-31-79

Title of Research Project: Characterization and Mode of Action of Phytotoxins produced by Alternaria dauci and other Alternaria spp. and their potential use in selection for disease resistant crop.

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Project's starting date: Jan. 1st, 1980.

Type of Report: Initial_____   Comprehensive_____   Final   x

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- 1) Alternaria 2) Phyto toxins 3) Plants - Diseases
and pest resistance

630.72 : 632.44; 581.29 :

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ABSTRACT

ALTERNARIA DAUCI - Zinniol is the major phytotoxin isolated from culture filtrates, mycelium and cell walls of A. dauci. Conditions for its optimal production in culture were investigated. Zinniol was detected during spore germination and early growth phases. The symptoms caused by zinniol were identical with those produced by the fungus. The structure-activity relationship was examined by comparing zinniol isomer and analogues derived from zinniol with zinniol for their phytotoxic effect on carrot. Evidence is presented for its metabolism by host leaf tissue.

ALTERNARIA HELIANTHI - Deoxyradicinol and deoxyradicinin were isolated from cultures of A. helianthi and Helianthus infected plants. The ability of A. helianthi to produce deoxyradicinin in liquid culture decreased after serial transfer, but was restored to the level of the original isolate in medium containing sunflower leaf extract.

ALTERNARIA CHRYSANTHEMI - A large yield of the phytotoxin radicinin has been identified in liquid cultures of A. chrysanthemi. The structurally related compound radicinol was also detected.

STEMPHYLIUM BOTRYOSUM - A novel phytotoxin - stemphyloxin I - was isolated from cultures of Stemphylium botryosum f. sp. lycopersici. This toxin ($C_{21}H_{34}O_6$) was crystallized and its structure determined to be a β -ketoaldehyde trans-decalin by X-ray crystallography. Stemphyloxin I is a highly unusual natural product. Injection of stemphyloxin I into a tomato leaflet caused unlimited necrotic spots and a loss of turgor which wilted the whole compound leaf. Stemphyloxin I is a non-specific toxin. It exhibits a differential toxicity towards various plants, tomato being the most sensitive. The relative phytotoxicity of stemphyloxin I was tested on cell suspension culture and leaf segments of various plants using (^{14}C) amino acids incorporation into protein as a bioassay. The highest sensitivity was detected with tomato cell suspension. Bioassay with leaf segments was approximately ten times less sensitive than with cell suspension. Attempts were made to select toxin-resistant tomato calli. Iron controls stemphyloxin I production. Its production was enhanced by the presence of succinate and glutamate in the medium.

Evaluation of the Research Achievements with Respect to the
Original Research Proposal

The main objective of the present proposal was to isolate and characterize phytotoxins produced by plant pathogens that might play a role in disease development. After achieving this goal it was proposed to initiate studies in the following directions :

(a) elucidate the mode of action of these toxins; (b) investigate the nature of differential toxicity of these toxins towards various plants and (c) attempts to select toxin-resistant calli from cell cultures. Alternaria dauci and other Alternaria spp. were originally chosen as the main pathogens because of the necrotrophic nature of their pathogenesis and availability of cell cultures of their respective host plants.

During the first year the Israeli group had concentrated on the characterization of phytotoxins produced by A. dauci and A. brassicicola as proposed in the original plan. In addition an extensive investigation was carried out on phytotoxins produced by the necrotrophic pathogen Stemphylium botryosum f. sp. lycopersici which had not been included in the proposal for the project. A novel and a potent phytotoxin, stemphyloxin I, was detected in culture filtrates of this fungus. S. botryosum f. sp. lycopersici is a serious foliage malady of tomatoes. Also, tomato plants have been successfully regenerated from protoplasts and tissue cultures. In these two respects S. botryosum is very similar to A. dauci and its host carrot, which were originally proposed as the main pathogen for the investigation. Our finding during the first year suggested that stemphyloxin I is much more a promising toxin for achieving the goals of the project than zinniol and other detected toxins from

Alternaria spp. Therefore, the efforts of the Israeli group in the last two years was shifted towards studies with stemphyloxin I, as indicated in the initial and comprehensive reports. Meanwhile the U.S. group continued to search for phytotoxins produced by other Alternaria spp. Novel phytotoxins produced by A. helianthi, the causal agent of leaf spot and seedling blight of sunflower and A. chrysanthemi, the etiological agent of a leaf spot disease of shasta daisy, were characterized.

One of the major achievements of the present project was the isolation of stemphyloxin I and elucidation of its structure by x-ray crystallography. Stemphyloxin I is a highly functionalized β -ketoaldehyde trans-decalin derivative. It does not closely resemble any previously reported metabolites of the plant kingdom. Furthermore, no other reported phytotoxin has a β -ketoaldehyde functionality. In fact, β -ketoaldehydes are quite rare among natural products. Stemphyloxin production appears to be iron regulated and it can form a chelate with Fe^{+++} to yield a colored product. Recognition in the uniqueness of the above mentioned findings was expressed by the forthcoming publication in "Science" which is now in press.

Another important achievement is the chemical characterization of radicinol and deoxyradicinol in culture filtrates of A. chrysanthemi and A. helianti respectively. This is the first report of the occurrence of these phytotoxins in the genus Alternaria. Here again, x-ray crystallography had been used for the structural determination of radicinin and radicinol. In addition to the three previously mentioned novel compounds, the known phytotoxins zinniol, radicinin and deoxyradicin were

identified by spectral procedures in A. dauci, A. chrysanthemi and A. helianthi respectively. Cultures of A. dauci also contained a low molecular weight glycopeptide (MW lower than 2000), which incited typical symptoms of Alternaria leaf blight in carrot. At least 2 phytotoxins have been detected in chloroform extracts of A. brassicola. However, since the last three compounds possessed low phytotoxicity they have not been chemically characterized.

During our studies special attention was paid to optimizing the growth conditions for production of the various phytotoxins. Thus, the presence of 4-carbon dicarboxylic acids, as well as iron favored stemphyloxin I secretion, whereas high carbon-nitrogen ratio in the presence of L-asparagine was required for optimal zinniol formation. Of special interest was the observation that the ability of A. helianthi to produce deoxyradicinin in liquid culture decreases after serial transfer on Czapek-Dox agar medium. The latter was restored to the level of the original isolate after transfer to medium containing sunflower leaf extract.

Studies on the structure-activity relationship was initiated with zinniol and stemphyloxin I. It was found, for instance, that the hydroxymethyl groups and isoprenoid chain were essential for the phytotoxic activity of the zinniol molecule. The enolic β -ketoaldehyde group of stemphyloxin I appears to confer biological activity to this toxin but additional functional groups might also contribute to its biological activity. Studies are now in progress to identify other functional groups responsible for toxicity.

Mode of action studies were carried out primarily with stemphyloxin I. The inhibitory effect of the toxin on exponentially growing tomato

green cells and white cells was similar, suggesting that the sensitive site for action of stemphyloxin I is either not specific to chloroplasts or absent in chloroplasts. Stemphyloxin I does not seem to disrupt membrane function since we could not detect either leakage of electrolytes from tomato leaf discs or inhibition of amino acids transport in the presence of the toxin. On the other hand, protein synthesis as measured by ^{14}C -amino acids was inhibited by stemphyloxin I. Whether the effect of stemphyloxin I on protein synthesis is direct or indirect is now under investigation.

All the investigated phytotoxins were non-specific but showed differential phytotoxicity on various plants. In the case of stemphyloxin I tomato, the host plant, showed the highest sensitivity, regardless of the bioassay procedure (e.g. injection or ^{14}C -amino acid incorporation) or the level of cell differentiation (e.g. leaves or cell cultures). It is noteworthy that production of deoxyradicin by A. helianthi was detected in leaves from naturally infected diseased sunflower leaves. This last fact is a supportive evidence for the role of the toxin in disease development. Although all ten accessions of Helianthus tested were sensitive to this toxin, quantitative differences between lines were noted.

Selection of stemphyloxin I calli clones from tomato cell cultures were attempted during the last year. We have encountered some technical difficulties in achieving this goal. Among them are the high percentage of escapees in the presence of the toxin. The breakdown of the toxin might be responsible in part to this problem. We are now in the process of characterizing the chemical structure of the breakdown product. We hope to obtain a stable toxic derivative of stemphyloxin I for use in selection experiments.

The presence of phytotoxins which can induce typical disease symptoms in culture filtrates does not necessarily imply involvement in pathogenicity. Nevertheless, the only promising way for identifying phytotoxins which participate in disease developments is to isolate and characterize them from culture filtrates and only then prove their involvement by genetic and biochemical means. Consequently, all our efforts were initially directed towards the isolation and structural determination of the phytotoxins produced by the selected pathogens. Only on this basis can investigations concerning the biological properties of these toxins in relation to the respective host plants be performed. The first stage, e.g. chemical characterization, has been successfully and completely achieved. Although many significant results related to the biological properties and mode of action have already been obtained, especially on stemphyloxin I, these studies, as well as the selection of toxin-resistant cell cultures, are long-term investigations. Therefore, this research is continued beyond the period of the project.

Description and Results of Co-operation

During the last 3 years we have exchanged ideas, results and discussions by frequent mail communication. Dr. Strobel visited Dr. Barash's laboratory during September 1980 for the purpose of reviewing results and planning further experiments. During 1982 Dr. Barash spent 5 months in Dr. Strobel's laboratory for common experiments and discussions. A direct result of this visit was the crystalization and structural determination of stemphyloxin I by x-ray analysis.

Benefit to Agriculture

- (a) The use of stemphyloxin I and possibly the other phytotoxins for screening disease-resistant plants ;
- (b) The identification of a new natural phytotoxic compound (stemphyloxin I) might be used, or lead to synthesis of new herbicides ;
- (c) Understanding the mode of pathogenesis of important pathogens.

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