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Contribution to the Physiology of *Trigonella* Infected with *Peronospora trifoliorum*

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Abstract. Some biochemical properties of *Trigonella foenum-groecum* infected with *Peronospora trifoliorum* have been investigated. Qualitative changes were observed in lipids, coumarins, amino acids and indole compounds only. A quantitative study of the phosphate content was also carried out. Neutral lipids did not show any significant changes. In addition to the fourteen phospholipid bands, the infected leaves contained a new band corresponding in R_f to phosphatidic acid or polyglycerophosphate. Seven coumarins were present in both the extracts. In addition 3-hydroxy-coumarin, 5-hydroxy-4-methylcoumarin and 7-hydroxy-4-methyl-coumarin were detected in infected leaves. Higher levels of indole acetic acid and three of its derivatives were observed due to infection. Infection altered the free amino acid content. Tryptophan appeared and the total phosphate content increased. Increase in phenols and indoles suggest a higher respiratory rate whereas a higher content of inorganic phosphate suggests uncoupling of phosphorylation from respiration.

Peronospora trifoliorum DE BARY is distributed widely on alfalfa (*Medicago sativa*) and clovers (*Melilotus* spp.) in the temperate zones of the world. This also infects species of *Astragalus, Lupinus, Medicago, Trifolium, Trigonella* and other related plants. It causes considerable damage to young plants and reduces stand of plants in the second year. In India it occurs mostly on *Trigonella foenum-groecum* L. and species of *Medicago* and *Melilotus*.

Peronospora trifoliorum occurs every year in a mild condition on leaves of *Trigonella foenum-groecum* in Delhi. But last year (1970) the disease incidence was very high due to a sharp fall of temperature and rise of air-humidity in the middle of January. The disease continued up to the third week of February with the same intensity and after that it receded. Generally in less severe conditions downy greyish-violet coloured sporangia are formed on the under surface of the leaves in patches of various sizes, sometimes covering most of the leaf surface (MUKERJI 1971). Upper surface of such leaves becomes discoloured, yellow to dull-green-oily, epidermal cells scattered over the underside collapse and chlorophyll disappears from the mesophyll. The infected tissues soon die and turn brown.

Last year due to severe infection of plants, downy growth also developed on the stem and influorescence and the leaves withered after two weeks. Such withered leaves contained oospores which are formed rarely.

Infection occurred when the plants were young; with maturity of the plants, the heavily infected leaves fell, but most of these plants once again regained their vigour in the month of March. But the yield in such plants was always reduced 50—60%.

It is known that an obligate pathogen affects the physiological activities of the infected plant. There is reduced photosynthetic activity because of the reduction of photosynthesis surface of the plant, through death (AGRIOS 1969). It is also known that there is increased host synthesis while the parasite is growing vegetatively and a general metabolite degradation during sporulation. But all the metabolites are not degraded during sporogenesis of the parasite. Some metabolites, such as starch, sugar, RNA and DNA decrease, others like proteins and amino acids increase while some others like lipids are not affected (WILLIAMS *et al.* 1968). In general there is an increase of respiratory rate (SHAW 1963).

There are several reports of biochemical investigations of the diseased tissue due to other members of downy mildew fungus. A review of literature revealed that there is no report of any work on the infected tissue of *Trigonella foenum-groecum* due to *Peronospora trifoliorum*. Advantage was taken of the severe and widespread infection last year for carrying out biochemical studies.

Materials and Methods

The healthy and infected leaves of *Trigonella foenum-groecum* were collected under sterile conditions from the plants grown in the experimental plots of the Botanical Garden of the University. Only leaves which were deep green and showed no signs of infection or physiological deficiency were collected for the sample of healthy leaves. That of diseased leaves consisted of leaves in the initial stages of infection and had sporangia on the lower surface. Highly infected necrotic leaves were not collected.

The lipids were extracted by grinding 5 g of fresh leaves with sand and 50 ml of chloroform : methanol (2 : 1 v/v). Proteolipids were dissociated by the method of FOLCH *et al.* (1957) and the final lipid extract was made up to a known volume in chloroform.

Phospholipids were separated by thin layer chromatography (TLC) on silica gel G (*E. Merck*) with chloroform : methanol : 7 M ammonium hydroxide (46 : 18 : 3, v/v/v) (ABRAMSON and BLECHER 1957). The phospholipid bands were made visible by exposing the plate to iodine vapours. Neutral lipids were separated by first running the plate in hexane : ether : acetic acid (90 : 10 : 1, v/v/v) and then in the same direction with the same solvent system in the ratio of 60 : 40 : 1 (v/v/v) (KATYAL *et al.* 1969).

The coumarins were extracted and analyzed by the method of MINAMIKAWA *et al.* (1963). 5 g of the leaves were homogenized with 50 ml of 95% methanol and immersed in a boiling water bath for 30 min. The extract was cooled and filtered, and the filtrate was evaporated under reduced pressure and made up to a known volume in methanol. The coumarins were separated by TLC with ethyl acetate : hexane : acetic acid (50 : 50 : 2, v/v/v) and their spots located by exposure to ultraviolet light.

The proteins of the leaves were hydrolyzed by refluxing 5 g of fresh leaves with 50 ml of 6 N HCl for 24 h. The acid in the filtrate was evaporated over a steam bath by repeatedly adding water to the residue and evaporating again. The residue was made upto a known volume in 10% n-propanol. The free amino acids were extracted by the method of DÉMÉTRIADES (1969) The amino acids were separated by two dimensional TLC (HIRS 1956).

The indole compounds were extracted from the leaves by ether and separated by the two dimensional TLC as described by STAHL and KALDEWEY (1961). The indole spots were observed by their fluorescence in UV after spraying with formaldehyde-hydrochloric acid reagent.

The total phosphate was extracted from the leaves by digestion with perchloric acid (ALLEN 1940) and was estimated by the method of NAKAMURA (1952). The inorganic phosphate was estimated after MUKERJEE and SHAW (1962): 50 mg of dry weight of leaves were homogenized in a blender with 20 ml cold 0·2 N perchloric acid and filtered through a Buchner funnel followed by repeated washing with water. The filtrate was made up to 100 ml and inorganic phosphate in a 10 ml aliquot of the filtrate was estimated by the method of NAKAMURA (1952). Organic phosphate was obtained by the difference between total and inorganic phosphates. Phospholipid phosphate was obtained by estimating phosphate in the lipid extract.

TLC was run in chromatoplates coated with 300 μm layers of silica gel G (*E. Merck*). The coated plates were activated for 1 h at 110° C except for the separation of indoles where activation was for 30 min. For the separation of amino acids, the plates were not activated. After running the plates, the required bands were eluted with methanol (*BDH, AR*) and their ultraviolet spectra were determined on a *Beckman Model DU* spectrophotometer. All reagents and solvents used were of *BDH* analytical reagent grade.

A visual comparison was made between the chromatograms of normal and infected leaves and all major differences were noted down.

Results

A number of biochemical parameters of normal and infected leaves were compared. The neutral lipid pattern was qualitatively similar in normal and infected leaves and the usual components such as mono-, di- and tri-glycerides, free fatty acids, sterols and sterol esters were present in both. Fourteen phospholipid bands were common to both normal and healthy leaves. Infected leaves exhibited a new phospholipids band which was absent in normal leaves. Its R_f corresponded to that of phosphatidic acid or polyglycerophosphate.

Seven coumarins were present in both the extracts from normal and infected leaves. In addition, the infected leaves contained three more coumarins with the following R_f values and ultra-violet absorption maxima (nm) — 0·086 (λmax 240, 325); 0·143 (λmax 250, 300); 0·34 (λmax 340). These values correspond to 3-hydroxy-coumarin, 5-hydroxy-4-methyl-coumarin and 7-hydroxy-4-methyl-coumarin respectively.

Five common indole compounds were observed on the chromatoplates. The infected leaves had a higher content of indoleacetic acid and also gave

rise to spots corresponding in R_f to indole-3-acetonitrile, indole-3-propionic acid and indole-3-butyric acid, which were absent in normal leaves.

Sixteen amino acids were detected in hydrolysates of leaves. There were no marked differences between normal and infected leaves. However, there was a difference in the free amino acid pattern. Leucine plus isoleucine and serine, glycine, glutamic acid, leucine and arginine were present in both types of leaves. The levels of serine, glycine, glutamic acid and leucine plus isoleucine were lower and that of arginine was higher in the infected leaves. On the other hand, tryptophan was present only in infected leaves.

TABLE 1

TOTAL PHOSPHATE CONTENT and various fractions in healthy and diseased leaves of *Trigonella*

Details	µg of phosphorus per 50 mg dry weight of the tissue	
	Healthy leaves	Diseased leaves
Total phosphate	834	1067
Inorganic phosphate	208	359
Organic phosphate	626	708
Phospholid phosphate	233	282
P inorg./P org. ratio	0·33	0·51

The infected leaves had a higher total phosphate content (Table 1). This increase was distributed among all the fractions including inorganic, organic and phospholipid phosphorus. The ratio of inorganic phosphate to organic phosphate was higher in infected leaves.

Discussion

Phosphatidic acid could probably have arisen by the breakdown of the host phospholipids or the phosphatidic acid or polyglycerophosphate could represent the phospholipids present in the fungal parasite. Many workers have reported an increase in the phenol content of the infected plant tissues. Accumulation of phenols has been observed by KIRALY and FARKAS (1962) in the stem rust disease of wheat due to *Puccinia graminis* and apple infected by *Venturia inequalis* and *Podosphera leucotricha* (BARNES and WILLIAMS 1960) and in the chocolate spot disease of beans due to *Botrytis cinerea* (DEVERALL 1961). The appearance of new coumarin compounds in infected leaves is in agreement with the above reports.

Higher levels of indole compounds observed in infected leaves are in accord with the findings of KIERMEYER (1958) in the case of white rust (*Albugo candida*) and *Peronospora parasitica* infected *Capsella bursa pastoris* as well as that of FEHRMANN (1965) in *Phytopathora* infected potato tubers.

The observed differences in free amin acids indicate a profound effect of infection by *Peronospora trifoliorum* on the amino acid metabolism of the host. It may be recalled that RUDOLPH (1963) reported similar changes

on the rust infected wheat. Tryptophan is precursor of indole compounds and its appearance in infected leaves is perhaps related to the increased synthesis of auxins.

During glycolysis and oxidative phosphorylation inorganic phosphate is converted into organic phosphate. The increase in the ratio of inorganic to organic phosphate in the infected leaves suggests that either glycolysis or phosphorylation is impaired on infection. Detailed studies of enzymes involved in these metabolic steps may provide valuable information.

Auxins influence a number of physiological processes including phenol metabolism and respiration (DALY *et al.* 1962, SEQUEIRA 1963). Increased auxin levels foster energy requiring processes leading to growth. Phenols have also been implicated in plant respiration (BONNER 1957). It is possible that the increase in phenols and auxins in the infected leaves increased the respiration of the leaves. But since the ratio of inorganic phosphate to organic phosphate increased in infected leaves there could have been uncoupling of phosphorylation from respiration (LATIES 1957).

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S. GOPAL, K. K. MAGGON, T. A. VENKITASUBRAMANIAN, K. G. MUKERJI, Universita Delhi, Indie: **Příspěvek k fysiologii rostlin *Trigonella foenum-groecum*, infikovaných houbou *Peronospora trifoliorum*.** — Biol. Plant. **13** : 396—401, 1971.

Byly vyšetřovány některé biochemické vlastnosti rostlin *Trigonella foenum-groecum* infikovaných houbou *Peronospora trifoliorum*. Kvalitativní změny byly pozorovány pouze v lipidech, kumarinech, aminokyselinách a indolových sloučeninách. Kvantitativně byl sledován též obsah fosfátů. V obsahu neutrálních lipidů nebyly pozorovány významné změny. Infikované listy obsahovaly kromě čtrnácti chromatografických zón náležejících fosfolipidům, ještě jednu zónu, jejíž R_f odpovídá kyselině fosfatidové nebo polyglycerofosfátu. V obou extraktech bylo zjištěno 7 kumarinů. V infikovaných listech byly stanoveny ještě 3-hydroxykumarin, 5-hydroxy-4-methylkumarin a 7-hydroxy-4-methylkumarin. Infekce působí zvýšení hladiny kyseliny indolyloctové a jejích tří derivátů. Také obsah volných aminokyselin se infekcí měnil: objevil se tryptofán a zvýšil se celkový obsah fosfátů. Vyšší obsah fenolů a indolových derivátů nasvědčuje zvýšené intensitě dýchání, zatímco vyšší obsah anorganického fosfátu ukazuje na to, že fosforylace není pravděpodobně napojena na dýchací procesy.