PRO EXPERIMENTIS

Mycoplasma-Like Organisms in Histological Sections of Infected Sandal Spike (*Santalum album* L.)

Both electron microscopy and treatment with tetracycline compounds[1] have provided evidence that spike disease of sandal (*Santalum album* L.) is caused by mycoplasma-like organisms. Further, HULL et al.[1] reported the complete absence of virus particles in diseased sandal but earlier workers[2,3] have reported the presence of intracelluar bodies in the parencymatous and epidermal tissues of spike-infected sandal plants. It has been claimed that these inclusions may be of viral origin[2,3].

Having in mind the recent investigations on mycoplasma diseases of plants and animals, we have tried to investigate the possible location of certain mycoplasma-like organismus or cytoplasmic inclusions of mycoplasmal origin by using certain specific stains and light microscopy.

Materials and methods. Procedure 1: Stems and leaves of healthy sandal plants and those of spiked plants in varying stages of infection were fixed in (FAA) formalin, acetic acid and alcohol in a ratio of 3:1:5 for 24 h and subsequently embedded in wax. Longitudinal sections of these materials were cut at 2 to 5 µm thickness, stained (1% aqueous Giemsa or Dienes stain) and mounted in canada balsam.

Procedure2: Freshly cut stems of the above materials were washed in warm phosphate buffered saline (pH 7.2) and fixed for 2–5 min in methyl alcohol. Longitudinal sections of these materials were cut at 2 to 8 µm thickness (using a sliding microtome), stained with Giemsa (0.1 g Giemsa in 100 ml phosphate-citrate buffer pH 6.8) for 2 h, then washed in 2 changes of acetone and 2 changes of a mixture of equal parts of acetone and xylol, cleared for 5 min in xylol and then mounted in canada balsam.

Results and discussion. Cytoplasmic inclusions were observed in the cells associated with phloem and xylem tissues (xylem parenchyma) of spiked sandal, whereas these bodies were not found in any tissues of uninfected plants. The cytoplasmic inclusions observed were not similar to those observed by earlier workers[2,3]. The inclusions stained darkly and appeared as spherical particles or branched chains of coccoid forms and were found distributed throughout the lumen of the cell, occupying nearly $^1/_3$ of the total cell volume or more in some cases. Dienes stain is generally used as a specific stain for mycoplasma-like organisms, but in these investigations, at least, Giemsa (Procedure 1) seemed to offer better staining.

Mycoplasma-like organisms have been cited in the phloem tissues by a number of investigators, but very rarely in the xylem and parenchymatous tissues[4].

HAYFLICK and STINEBRING[5] have reported the presence of purple coloured bodies of pleuropneumonia-like organisms (PPLO) in chick embryo tissues. These bodies stained deeply with the May-Grunwald-Giemsa stain. They were called intracytoplasmic inclusions, comparable to 'elementary bodies' (smallest unit of a mycoplasma) or groups of them. Similar aggregated inclusion bodies of PPLO have been reported[6] in the cytoplasm of HeLa cell cultures. These bodies were found scattered throughout the cytoplasm in necrotic cells. WHITTLESTONE[7] again using Giemsa stain, has observed colonies consisting of branching chains of coccoid globular structures in pig lung tissues infected by mycoplasma-like organisms. BAWYER[8], using electronmicroscopy, has reported spherical mycoplasma-like organisms in longitudinal sections of 'Legume Little Leaf'.

The intracytoplasmic spherical bodies observed in the infected sandal were indentical in structure with the bodies observed by HAYFLICK and STINEBRING[5] and by BAWYER[8]. The colonies of coccoid forms, which often showed connections with one another, were identical with those observed by WHITTLESTONE[7] in animal tissues. The morphology of these inclusions is so distinctive that they are almost certainly of mycoplasmal origin. They could either

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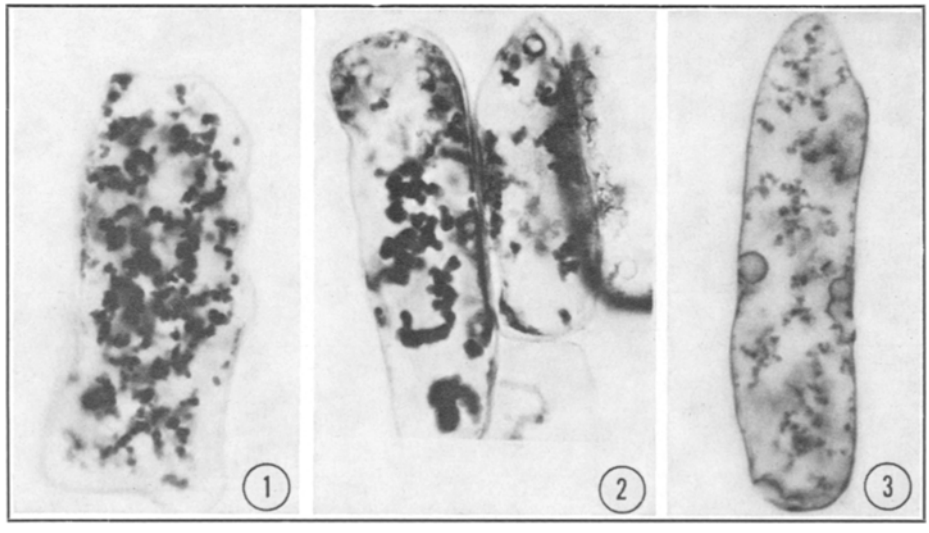

Fig. 1, 2 and 3. Parenchymatous cells (phloem) showing cytoplasmic inclusions of mycoplasmal origin in the spike infected sandal plants. × 3500.

be mycoplasm-like organisms or products of their metabolism, or both.

If these observations are validated, then they could provide a simple method for preliminary screening of infected tissues, particularly over large areas.

Résumé. Une méthode simple est décrite pour l'étude radioscopique des tissus du bois de Santal (*Santalum album* L.) infectés par un organisme du type dit mycoplasme. Les techniques comprennent au préalable l'emploi de colorants Giemsa et Dienes, précédant l'étude microscopique. Des organismes du type dit mycoplasme et (ou) des inclusions cytoplasmiques d'origne mycoplasmique ont été localisées dans les tissus contaminés du bois de Santal, surtout dans les régions du xylème et du phloème.

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The Amylolysis of a Substituted Starch Substrate

Although a large number of procedures for the assay of α- or β-amylase are reported in the literature, the separate measurement of the activities of these enzymes in the same reaction medium still poses a problem in investigations dealing with the occurrence of α- and β-amylase isozymes in germinating seeds or when studying applications in both industrial fermentation processes and in food and pharmaceutical industry. Preliminary experiments based on a controlled selective denaturation of one of the enzymatic partners in the reaction mixture have not given us satisfying results.

The difference of rheology [1] during the α- or β-amylolysis does not allow discrimination between α- and β-amylolytic activities, since viscosimetric measurements follow the variation of the weight average degree of polymerization of the substrate and the reaction products, while the expression of the hydrolytic activity is a function of the numerical average degree of polymerization. It is obvious that the variation of these two parameters during the endohydrolysis by α-amylase or the exohydrolytic attack by β-amylase does not proceed in the same manner, so that it is difficult to establish in both cases a relationship between a drop in viscosity and the hydrolase-activity.

We found, however, that by coupling, for instance, a chromogenic molecule to starch, the exohydrolytic action of β-amylase on this substrate is inhibited, while these substituents on the helixes of the substrate only function as accidental barriers for α-amylase and do not hinder the progressing endohydrolytic attack. To find a suited substituent was not so difficult, since chromogenic substances which can be bound covalently with the glucose monomers, were developed for the textile-industry. For this purpose the Cibachron Blue F3GA (Ciba), a chromogenic product of the monochlorotriazine type, proved to be indicated.

Material and methods. Crystalline α-amylase from hog pancreas and β-amylase from sweet potatoes are prepared by the Worthington Biochemical Corporation. Dilutions are made with a solution containing 0.1% ovalbumin (Sigma Grade V), 16% NaCl and 0.05% $CaCl_2.2H_2O$. The measurement of the total α- and β-amylolytic activity is performed by means of a reductometric method [2], with starch after Zulkowsky (Merck) as substrate and 3,5-dinitrosalicylic acid as oxydizing agent. The assay of the α-amylase activity using a chromophore substrate (Phadebas, Pharmacia) is conducted by the colorimetric procedure [3] or by the plate diffusion technique [4] described by CEŠKA.

Results and discussion. The influence of a substitution starch substrate on the exo- and endo-amylolytic attack can very well be evaluated semiquantitatively with the help of a plate diffusion technique by incorporation in the agargel of a suitable chromophore (Cibachron F3GA) starch preparation. We find that only α-amylase, due to its endoamylolytic activity, produces a well defined circular transparent digestion zone, while the presence of β-amylase, even in high amounts (90% w/w), does not give a visible effect. We could also differentiate the α- and β-amylolytic activities quantitatively in the same preparation (Table). The total α- and β-amylase activity, in nmoles glucosidic bonds hydrolysed per min, is measured with a reductometric method, while the specific colorimetric dosage of the α-amylase activity is performed by means of an insoluble chromogen coupled substrate. The

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Differentiation of α- and β-amylolytic action

Enzyme per assay (ng)		α-amylase activity (chromophore substr.)	Total α- and β-amylase activity (reductometric)	β-amylase activity
α-amylase	β-amylase	(A_{620} converted to nmol/min^{-1})	(nmol/min^{-1})	(nmol/min^{-1})
0	9	0	27	27
18	9	13	40	27
36	9	26	53	27
54	9	39	66	27

To evaluate the α-amylase activity in nmol/min^{-1}, the absorbance values measured using the chromophore substrate, are multiplied by 0.23.