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# The occurrence of inclusion bodies in leaf epidermis cells of sandal affected with spike disease

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**Abstract**

Round or slightly oval inclusion bodies were found in low numbers in epidermal cells of spiked (expressing spike disease symptoms) leaves. They could not be demonstrated in epidermal tissue of apparently healthy leaves. The intracellular inclusions stained with phloxine and rose bengal. Their relationship to sandal spike virus is discussed.

## Introduction

Spike disease is a serious virus disease of the sandal (*Santalum album* L.) which was first reported in South India in 1899 (Coleman, 1917; Venkata Rao and Gopalaiyengar, 1934; Rønde Kristensen, 1960).
The disease causes a diminution of the size of the leaf which becomes very narrow due to the fact that the decrease in breadth is much greater than that in length (Fig. 1). The affected leaves stand out stiffly from the branch, which acquires a spike-like appearance, and become crowded on the leaf-bearing branches as a result of a shortening of the internodes. One of the first symptoms that can be observed consists of phyllody of the flowers, causing a marked reduction of the perianth and stamens, accompanied by a leaf-like enlargement of the pistil. At the onset of the disease only parts of the tree may show the above-mentioned symptoms but gradually the entire tree becomes affected. Within two or three years after infection the tree dies.
Intracellular bodies were found to be associated with the spike disease by Narasimhan (1928, 1933). According to this author, microtome sections of spiked sandal leaves showed the presence of these intracellular bodies in almost every cell. However, the data presented by him (Narasimhan, 1933) refer only to parenchyma tissue of leaves and petioles.
Having in mind recent investigations on other virus diseases, e.g. dahlia mosaic (Robb, 1963, 1964), in which the epidermis of infected plants proved to be a rich source of intracellular (inclusion) bodies, epidermal tissue was examined from sandal leaves affected with spike disease.

Fig. 1. Partially spiked branch of a sandal tree affected with spike disease. H = healthy-looking leaves; S = "suspicious" leaves; Sp = leaves expressing spike symptoms.

*Fig. 1. Gedeeltelijk "spike" vertonende tak van een sandelboom, aangetast door de "spike"-ziekte. H = gezond uitziende bladeren; S = "verdachte" bladeren; Sp = "spike" vertonende bladeren.*

## Materials and methods

Spiked sandal material was collected from naturally infected areas in Kenchenhalli (near Bangalore) and Chamundi Hill (Mysore). In order to get some insight into the distribution of possible inclusion bodies, heavily spiked leaves from three fully spiked trees as well as healthy looking and spiked leaves from two partially spiked trees were used for the investigations. Two healthy sandal seedlings grown in the nursery of the Forest Research Laboratory (Bangalore) and kindly supplied by Dr P. S. Rao, Director of this laboratory, served as control. An attempt was made to remove portions of epidermis from the leaves by forceps. As it proved to be impossible to separate either upper or lower epidermis in this way from the rest of the leaf, because both epidermal layers were too tightly connected with adjacent cell layers, the upper epidermis was isolated by carefully scraping off the other tissues of the leaf by forceps.
The following stains and staining techniques were used.
*Phloxine.* Epidermal tissue was immersed briefly in 1 % NaCl, stained in a solution of 1 % phloxine in 1 % NaCl for 15 min, rinsed and mounted in 1 % NaCl. The technique is that of Robb (1963) except that the staining period was longer.
*Rose bengal.* Epidermal tissue was fixed in 8 % formalin for 15 min, stained in a solution of 0.5 % rose bengal for 15–25 min, rinsed and mounted in distilled water.
*Trypan blue.* Same technique as mentioned for rose bengal.
*Neutral red.* Epidermal tissue was immersed briefly in 0.5 % neutral red, rinsed and mounted in distilled water.
*Methyl green–pyronin.* Methyl green (0.5 g in 100 ml. 0.1 M acetate buffer pH 4.4) was extracted with chloroform to remove methyl violet; 0.4 g pyronin G (or 0.2 g

pyronin B) was then dissolved in this solution. Epidermal tissue was immersed in this solution for 5–10 min, rinsed and mounted in distilled water. The technique is that of Taft (1951) and Robb (1964) except that pyronin G was used in higher concentration. Staining with methyl green–pyronin proved to be unsatisfactory; no differentiation could be achieved between nucleus, nucleolus and other cell components whereas the cytoplasm stained bright reddish-blue. Trypan blue and neutral red gave only slight differentiation between nucleus and cytoplasm. Staining with phloxine and rose bengal was found to give the best results.

**Results**

Inclusion bodies were found in approximately 1 % of the epidermal cells in most of the preparations of heavily spiked leaves as well as in those of suspicious-looking (curled and yellowish) leaves of partially spiked trees (Fig. 2A–F). The inclusions were not evenly distributed over the cells, but appeared mostly in clusters of three or more cells. They could be demonstrated neither in epidermal cells of leaves of healthy trees nor in those of healthy-looking leaves of partially spiked trees, about 50 preparations of each of which were examined. Usually the inclusion bodies stained the same bright red with phloxine and bright pink with rose bengal as the nucleus, but in some preparations they were slightly lighter in colour. They were generally round or somewhat

Fig. 2 A-D. Epidermal tissue of "spike"-affected sandal leaves, stained with 1 % phloxine. In most of the preparations the inclusion bodies (arrowed) stained in the same manner as the nucleus (N).

*Fig. 2. A-D. Epidermisweefsel van "spike" vertonende bladeren van de sandelboom, gekleurd met 1% floxine. In de meeste preparaten waren de insluitsels (pijltjes) even sterk gekleurd als de kern (N).*

oval with a granular appearance and their size was variable, ranging from 2–7μ, but more often similar to that of the nucleus (3.5–7μ). Sometimes they were found in close proximity to the nucleus but no direct relationship could be observed between the two.

## Discussion

The inclusion bodies described here do not resemble at all those described by Narasimhan (1933). The intracellular bodies observed by him were much bigger than the nucleus and appeared mostly in close juxtaposition with the latter; they had a reticulate, highly vacuolate structure and sometimes even pseudopod-like proliferations. Further, they were present in nearly all the cells in the sections of spiked leaves, whereas in the present investigation only 1% of the cells contained one. However, Narasimhan's findings only held for parenchyma tissue of leaves and petioles so that the discrepancy between his results and those of the present author might be explained by the fact that different tissues were investigated. Although the inclusion bodies could only be observed in leaves of trees affected with the spike disease, there is still no conclusive evidence that there is a correlation between the sandal spike virus and the occurrence of these inclusion bodies. The possibility cannot be excluded that a second, possibly "latent" virus, unrelated to spike virus, is involved, being responsible for the formation of inclusion bodies in epidermal cells and parenchyma tissue. The fact that inclusion bodies could not be demonstrated in apparently healthy leaves does not necessarily contradict this hypothesis; it might be ascribed to escape, due to the difference between healthy-looking and spiked leaves in their cell contents, the former containing a large number of chloroplasts (in parenchyma tissue) and oil drops (in epidermal tissue) by which detection of inclusion bodies might have been obscured. Furthermore, it should be emphasized that preparations from spiked leaves did not always reveal the presence of inclusion bodies in the epidermis; they could be established in approximately 350 out of the 500 preparations examined.

McWhorter (1965) considers that the presence of specific inclusions in plant cells proves the presence of a certain virus. The question whether the inclusion bodies discussed in this paper are formed by the sandal spike virus or by some other, possibly "latent" virus occurring in sandal can only be answered after isolation of the sandal spike virus and successful transmission of the purified virus to an absolutely virus-free sandal seedling.

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## Samenvatting

*Het voorkomen van insluitsels in de epidermiscellen van bladeren van de sandelboom, aangetast door de "spike"-ziekte*

De "spike"-ziekte is een ernstige aantasting van de sandelboom *(Santalum album* L.) welke in 1899 voor het eerst in Z.-India is waargenomen. De symptomen die door deze ziekte worden veroorzaakt, bestaan uit het korter worden van de internodiën, het kleiner worden van de bladeren waarbij de groei in de breedte sterker geremd wordt dan die in de lengte zodat de zieke bladeren relatief langer zijn dan de gezonde, en het stijf van de tak gaan afstaan van de smalle bladeren, waardoor de twijg een aar ("spike")-achtig uiterlijk krijgt (Fig. 1). Tevens treedt vaak in het beginstadium van de ziekte fyllodie van de bloemen op, bestaande uit een sterke reductie van het bloemdek en de meeldraden en het bladachtig uitgroeien van de stamper. Twee tot drie jaar na de infectie sterft de boom. Uit microscopisch onderzoek bleek, dat insluitsels in kleine aantallen voorkwamen in de epidermis van uitgesproken "spike" vertonende bladeren alsmede in die van "verdachte" (enigszins vergeelde, krullende) bladeren aan dezelfde tak, doch niet in de gezond uitziende bladeren aan deze tak (Fig. 2A–F). Het aantal cellen, dat zo'n insluitsel bezat, bedroeg ongeveer 1 % van het totale aantal epidermiscellen in het preparaat. Opvallend was het, dat de insluitsels niet regelmatig over de cellen waren verdeeld, doch meestal in groepjes van drie of meer cellen voorkwamen. De celinsluitsels hadden een enigszins korrelige structuur en kleurden met floxine en bengaals roze even donkerrood respectievelijk donkerroze als de kern, of iets lichter.

Hoewel de insluitsels alleen waargenomen zijn in "spike" vertonende bladeren is het toch nog niet bewezen, dat er een samenhang bestaat tussen het "sandal spike"-virus en het optreden van de insluitsels. Hierover kan pas zekerheid worden verkregen, zodra men erin is geslaagd het "sandal spike"-virus te isoleren en met het gezuiverde preparaat een virusvrije sandelboom te infecteren.


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