The Root-Nodules of the Cycadaceae.

BY

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With Plate XXIX.

THE Cycadaceae are a group of special interest, owing to their retention of a greater number of primitive characters than are possessed by any other living group of Gymnosperms. Representatives of this group probably existed during the Mesozoic Period and were very widely distributed. The living cycads are represented by five eastern genera: *Cycas*, *Encephalartos*, *Stangeria*, *Macrozamia*, and *Bowenia*, and four belonging to the western hemisphere: *Zamia*, *Microcycas*, *Dioon*, and *Ceratozamia*, all of which are tropical or subtropical. In all the genera with the exception of the monotypic genus *Microcycas*, which is confined to Cuba, and has not been examined by the author, there are in addition to the primary tap-root numerous small secondary roots, some of which form the so-called root-nodules.

These structures have been described as 'Coralline roots' in *Cycas*, where they occur abundantly immediately below the surface of the soil and protruding above it. Reinke described them as special organs for aeration, Schneider suggested a symbiotic association, and Life isolated from them an Alga and some Bacteria, and believed them to be concerned in the assimilation of nitrogen as well as aeration. More recently Zach states that they are not symbiotic, but contain a fungal parasite against which the cells react as a phagocyte.

Amongst non-leguminous plants it is now recognized that the Elaeagnaceae, Myricaceae, Podocarpaceae, and the genus *Alnus* have root-nodules, which are definitely concerned with nitrogen assimilation. With these the Cycadaceae must be associated, because Bottomley has isolated from the nodules of *Cycas* not only *Bacillus radiculicola* but also *Azotobacter*, both of which organisms are known to assimilate atmospheric nitrogen. They are, however, of special interest because in their cortex a very definite green ring, the algal zone, is produced by the presence of an *Anabaena*, which has been described by the author.

The first part of the present series of investigations was carried out with material of *Cycas circinalis* and *Encephalartos Hildebrandtii* kindly supplied by the Curator of Chelsea Physic Gardens; and was extended to comprise the genera *Stangeria*, *Macrozamia*, *Zamia*, *Ceratozamia*, *Dioon*, and *Bowenia*, through the kindness of the Director of the Royal Botanic Gardens, Kew, both of whom I desire to thank.

Root-nodules have been found to occur throughout all the cycadean genera, and as in other non-leguminous plants they are perennial modified lateral roots which have diverged from their normal growth owing to their having been infected with the nitrogen-fixing organism *Bacillus radicicola*.

In *Cycas circinalis* they are confined to the surface of the soil and the layers immediately below. It has, however, been ascertained that the lower roots frequently become infected with *Bacillus radicicola*, which penetrates the root-hairs and enters the cortex, where its presence stimulates the root to become negatively geotropic (Fig. 1), and when near the surface of the soil the tip becomes swollen and subsequently profusely branched, giving rise to a coralloid mass (Fig. 2). Large masses of these structures are visible on the surface of the soil, and they characteristically contain a very definite green ring, the algal zone. The same conditions occur in *C. revoluta* and *C. seemanni*, but some species in cultivation at Kew have not developed these nodules very abundantly.

Encephalartos Hildebrandtii and all the species cultivated at Kew exhibit a similar phenomenon, but the individual nodules are larger, and thus, although the branching is very profuse, the clusters are less dense (Fig. 3).

Examination of a large number of nodules from both *Cycas* and *Encephalartos* showed that the nodule tip may become quite large and even branched several times, while no algal zone is present (Fig. 3, *a*, *b*). The Alga therefore lives in the soil, and must at some period gain an entrance to the nodule. The roots which have become negatively geotropic have *Bacillus radicicola* in their cortical cells, but they retain their normal appearance. Very soon, however, a ring of structures, apparently lenticels, is formed a little distance from the tip, which subsequently becomes swollen (Figs. 1 and 3 *a*). The lenticels consist of large masses of very loosely arranged parenchymatous cells formed from the phellogen. They soon become infected with *Bacillus radicicola* and also *Azotobacter*, the latter at first making their way between the cells, and later entering them (Fig. 9). Gradually in the nodule, i. e. the portion above the ring of lenticels, the outer cells produced by the phellogen increase in size owing to infection with *Bacillus radicicola*, and are pushed apart by masses of *Azotobacter*, some of which subsequently penetrate into them, with the result that there is a zone on the outside of the nodule which contains the two nitrogen-fixing organisms associated together (Fig. 9). This zone extends from the basal lenticels

nearly to the meristematic apex. In many cases *Anabaena* filaments are associated with these large loose outer cells, especially in the angles produced by the nodule branching (Fig. 9).

Other nodules have a very definite green algal zone traversing a corresponding area of the nodule, but enclosed by a few layers of cells (Fig. 11). The three organisms *Bacillus radiculicola*, *Azotobacter*, and *Anabaena* now all become concentrated in this area, which consists of a large air space inhabited by the *Anabaena* and *Azotobacter*, and traversed by elongated papillate cells connected with both the inner and outer tissues, thus keeping the zone intact, and being themselves rich in protoplasmic contents, and containing *Bacillus radiculicola* (Fig. 10). The cortical cells between the algal zone and stele are remarkably free from infection, although some contain the two Bacteria, most of them are filled with starch grains or calcium oxalate crystals. Outside the algal zone is a very definite phellogen which produces a few layers of parenchymatous cells towards the zone, and a few very regular cells on the outside. The algal zone always begins very abruptly immediately above a lenticel, but at the apex of the nodule the *Anabaena* appear to make their way gradually between the cells into which also the Bacteria penetrate, thus continuing the growth of the zone with that of the nodule. Sometimes, however, it is localized to quite a small area, perhaps on one side of the nodule. Associated with its production is an extended development of lenticels at some portion of which the zone is always interrupted (Fig. 11). In nodules without an algal zone only a basal ring of lenticels occurs, but where it is present they are produced in connexion with every branch and at quite frequent intervals on the branches themselves (Fig. 3). The nodule often branches dichotomously, but lateral branches also arise, particularly beneath the primary ring of lenticels, which may become nodules or lateral roots (Fig. 5).

Whilst visiting Kew Gardens, one of the *Stangeria Schizodon* plants was observed to have a piece broken from the pot quite near the bottom, and from this hole a number of roots upon which were numerous small nodules were seen to be emerging. This aroused the question whether the nodules in other members of the Cycadaceae were confined to the surface of the soil or not. Their formation here might have been induced by the exposure to light and air resulting from the presence of the hole, or it might be that they were more or less uniformly distributed throughout the pot. On inquiry the latter was found to be the case.

In *Stangeria* small nodules are produced all along the root, each of which branches dichotomously, and thus little clusters are formed (Fig. 4). Infection of the root with *Bacillus radiculicola* occurs through the root-hairs, and these organisms infect the young lateral roots whilst they are traversing the cortex, and thus their growth in length is arrested, and they are stimulated to branch almost immediately they are free from the parent

root. The root-tip may also develop a ring of lenticels and form the beginning of a cluster of nodules. At the base of the nodules which are produced laterally the phellogen becomes very active, and gives rise to a complete ring of lenticellular tissue (Fig. 12). Between this basal ring of tissue and the nodule large groups of *Azotobacter* and sometimes *Anabaena* collect, both of which are also associated with the outer cells of the nodule, as in *Cycas* and *Encephalartos*. As the nodule develops, other lenticellular areas may appear, which are very out-curved with a strong tendency to form a zone of parenchyma parallel with the surface of the nodule, and frequently small localized areas, containing the three organisms so characteristically associated with the outer cells, become completely enclosed (Fig. 13).

Nodules in which an algal zone is developed become very much elongated, always remaining very slender, and branching repeatedly (Fig. 4, *b*). In these nodules the zone occupies a larger proportion of the cortex, and is traversed by many more papillate cells than in *Cycas* and *Encephalartos*. The three symbiotic organisms extend through the outer cortex to the phellogen usually, and are often associated with the outer cells. Nodules have been examined which had enclosed, in addition to an algal zone, certain other areas of Algae and Bacteria on the surface. In branched nodules the two algal zones of respective branches have been found separated from one another by a band of meristematic tissue.

The nodules of *Macrozamia Macleyi* (Fig. 6) and *M. Dennisoni* resemble very closely those of *Encephalartos* in which no algal zone has developed, and no algal zone has been found in these nodules. Correlated with this is the scarcity of nodules on the surface of the soil in the plants at Kew. Their development corresponds with that described above, but the outer cells, in which *Azotobacter* and *Bacillus radicola* are found together, are so large and loosely arranged that the surface of the nodule presents an uneven spongy appearance. In some cases the infection with *Azotobacter* extends well into the cortex throughout which *Bacillus radicola* is distributed.

In *Ceratozamia mexicana* (Fig. 7), and *Zamia lindeni* (Fig. 5), the nodules observed remain quite small although branched, and are arranged very regularly in two rows along the root. In *Ceratozamia* large numbers are visible on the surface of the soil. A few roots of *Bowenia spectabilis* were also obtained upon which were some very young nodules. In these three genera *Bacillus radicola* is present in the cortical cells of the root and nodule. The basal phellogen produces an envelope of parenchymatous tissue as in *Stangeria*, and in some of the older nodules a second phellogen appears in that area inside the old one. The loose outer cells usually have *Azotobacter* associated with them, but no Alga has been found present in these genera.

The roots of *Dioon spinulosum* and *Dioon edule* produce successive

layers of cork which gradually become split and peel off. The cells themselves are rich in tannin. In addition to, and perhaps correlated with, this somewhat extensive development of periderm, large fan-shaped masses of parenchyma are produced at frequent intervals on the roots. There is a greater development of cells resembling lenticellular complementary cells than has been observed elsewhere. Large masses are present at the base of all lateral roots and also nodules (Fig. 14), which are abundant on roots a little below the surface of the soil. The nodules are repeatedly branched; in some cases two rows of small branches are produced from a central portion, but eventually large spherical masses are produced (Fig. 8). These small nodules show the typical structure described above where no algal zone is present. In addition to these, very much enlarged root-tips are visible on the surface of the soil, which also contain *Bacillus radicola* and have a thick periderm in which numerous very large lenticels are developed. One nodule was obtained which had a definite algal zone (Fig. 8, *b*) occupying a large area. Since only those nodules, to which the sun's rays can penetrate, can contain a living green organism, it seems probable that it is in the swollen tips which reach the surface that the *Anabaena* finds its home.

From these investigations the conclusions have been reached that root-nodules are universally present throughout the Cycadaceae, and they are primarily produced by infection with *Bacillus radicola*, and are therefore concerned with nitrogen assimilation. They are adapted to utilize both the nitrogen-fixing organisms *Bacillus radicola* and *Azotobacter* which are known to fix a greater quantity of atmospheric nitrogen per unit of carbohydrate when growing together than separately.

There is also sometimes a fourth symbiont present—the *Anabaena*. It is not, however, essential to the formation of the nodule, since in *Macrozamia*, *Zamia*, *Ceratozamia*, and *Bowenia* it has not been found, and only in one instance in *Dioon*. The presence of lenticels is evidently correlated with the formation of the algal zone, since they are much more abundant on nodules in which it is developed, and the zone itself is always interrupted immediately below some part of the lenticel. These facts, together with the universal occurrence of a ring of lenticels, or a basal ring of parenchyma with an active phellogen, and the presence of *Azotobacter* and *Anabaena* amongst the outermost layers of cells, suggest very strongly that the outer layers of the original nodule become broken down to form the algal zone, and the phellogen of the basal and lenticellular areas is stimulated by the presence of the *Anabaena* to develop a zone of cells, parallel with the surface of the nodule, which gradually extends and forms a protective layer. This is supported by (*a*) the formation of the local enclosed areas on the surface of the nodule, containing the three organisms in *Stangeria*; (*b*) the algal zone retaining a localized position in the nodule which has been observed in *Cycas*, *Encephalartos*, and *Stangeria*, and also

(c) the separation of two algal zones by a meristem which has been observed in *Stangeria*.

The *Anabaena* would naturally confine itself to an area not many cells deep, if photosynthesis were to go on, because of its dependence on light, the intensity of which must be rapidly diminished as it passes through the overlying cells. It is also impossible for the algal zone to be developed in nodules situated far below the surface of the soil, to which the photosynthetic rays of the sun cannot penetrate. In those cases in which the algal zone appears to be entirely absent it may be due to the method of cultivation, possibly to the absence of the specific *Anabaena* from the soil, since nodules are produced so abundantly without it and correspond very closely with those of other genera in which it afterwards develops. The algal zone is an area in which photosynthesis is taking place and probably attracts chemotactically the nitrogen-fixing organisms which require a carbohydrate as their source of energy. The three organisms thus collect together and work symbiotically, undoubtedly benefiting the cycad by the rich supply of elaborated material they produce.

In conclusion my thanks are due to Professor W. B. Bottomley for his sympathy and suggestions during the progress of these investigations.

SUMMARY.

1. All the cycadean genera produce root-nodules which are perennial, modified lateral roots, repeatedly branched and typically forming large coralloid masses.
2. They are produced primarily by infection with *Bacillus radicicola*.
3. A whorl of lenticels or a continuous zone of loosely arranged parenchymatous cells is produced at the base of each nodule.
4. The outer cells always become pushed apart and infected by *Azotobacter* and, if suitable conditions prevail, by *Anabaena* also.
5. The presence of the Alga stimulates the phellogen to produce other lenticels, from which and the basal area a zone of tissue is produced which encloses the original outer cells in which are the Alga and Bacteria.
6. The algal zone is continuous, except immediately below the lenticels, extending from the base nearly to the meristematic apex.
7. The algal zone consists of a large air-space containing *Anabaena* and *Azotobacter* which is kept intact by papillate cells traversing it from both the inner and outer tissues.
8. *Bacillus radicicola* is chemotactically attracted to the algal zone; thus leaving the cortical cells in which large quantities of starch grains and sphaeraphides are deposited, and in *Dioon*, also tannin.
9. No algal zone has been observed in *Macrozamia*, *Zamia*, *Ceratozamia*, and *Bowenia*, but nodules are produced containing *Bacillus radicicola* and *Azotobacter*.

10. The Cycadaceae, a group with many primitive characters, are the only nodule-bearing plants known, in which four organisms are associated together symbiotically, viz. two nitrogen-fixing Bacteria, an Alga, and the Cycad.

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DESCRIPTION OF PLATE XXIX.

l = lenticels; *r. t.* = root-tip; *r.* = root; *n.* = nodule; *v. b.* = stele; *g. p.* = meristematic apex; *p.* = phellogen; *f.* = cortex; *b.* = cells containing bacteria; *a.* = loose outer cells; *c.* = periderm; *B. r.* = *Bacillus radicola*; *Az.* = *Azotobacter*; *An.* = *Anabaena*; *s.* = starch; *o.* = calcium oxalate.

Fig. 1. Roots of *Cycas circinalis*, some (*n.*) negatively geotropic owing to infection with *Bacillus radicola*. Nat. size.

Fig. 2. Root-nodules of *Cycas circinalis*. Nat. size.

Fig. 3. Root-nodules of *Encephalartos Hildebrandtii*. Nat. size. (*a*) large tip without an algal zone; (*b*) branched nodule without an algal zone; (*c*) cluster with zone developed. Nat. size.

Fig. 4. Root-nodules of *Stangeria Schizodon* (*a*) without algal zone; (*b*) with algal zone. Nat. size.

Fig. 5. Root-nodules of *Zamia Lindenii*. Nat. size.

Fig. 6. Root-nodules of *Macrozamia Macleyi*. Nat. size.

Fig. 7. Root-nodules of *Ceratozamia mexicana*. Nat. size.

Fig. 8. Root-nodules of *Dioon spinulosum* (a) spherical masses and large swollen tip; (b) with algal zone present. Nat. size.

Fig. 9. Part of longitudinal section of nodule of *Encephalartos* without an algal zone. $\times 70$.

Fig. 10. Portion of the algal zone of the nodule of *Encephalartos*. *p.* = papillate cells. *n.* = nucleus. $\times 325$.

Fig. 11. Part of longitudinal section of nodule of *Encephalartos* with an algal zone. *g.* = gap in algal zone by lenticel; *E.* = end of algal zone. *A. Z.* algal zone. $\times 35$.

Fig. 12. Part of longitudinal section of nodule of *Stangeria* showing the basal ridge of tissue = *b. r.* $\times 35$.

Fig. 13. Portion of a longitudinal section of nodule of *Stangeria* with enclosed areas of Bacteria and Algae. $\times 70$.

Fig. 14. Part of longitudinal section of root and nodule of *Dioon*, showing mass of fan-shaped parenchyma = *b.* at base of nodule; *m.* = meristematic tissue; *t.* = tannin. $\times 35$.

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