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THE RELATION BETWEEN SEEDS AND MICRO-ORGANISMS.

BY

T. R. Sathe and V. Subrahmanyam.

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DR. M. O. FORSTER, F.R.S., CHAIRMAN OF EDITORIAL BOARD.

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DR. M. O. FORSTER, F.R.S., CHAIRMAN OF EDITORIAL BOARD.

## THE RELATION BETWEEN SEEDS AND MICRO-ORGANISMS.

By T. R. Sathe and V. Subrahmanyam.

Although it is generally recognised that all seeds germinate under favourable conditions of temperature, moisture, and substrate, the various changes by which an apparently inert seed becomes a growing plant remain obscure. The early softening of the seed-coat, absorption of water and consequent swelling are essentially physical and can be readily followed : but the nature of the forces that link these with a succession of processes such as respiration of the seed, development of various enzymes and emergence of the seedling, forms one of the most fundamental and elusive problems in the study of plant-life.

The natural substrate for germination is the soil and the large number and variety of soil micro-flora led several of the earlier workers to suggest that the first stage in germination is induced by micro-organisms, which it was believed were responsible for the transformation of nutrients in the seed and the growth of the seedling. The study of the relation between the micro-organisms of the soil and the germinating seed or the growing plant has, however, been hindered by the fact that conditions suitable for vegetative growth are also favourable to the active functioning of microscopic life : proper conditions of temperature and moisture and the occurrence of useful amounts of decaying organic matter help the development of plants as well as micro-organisms and, in absence of any evidence to the contrary, it has been generally assumed that micro-organisms are necessary for the germination of seed and the development of seedlings.

From his studies on putrefactive microbes Duclaux inferred that micro-organisms are essential to germination ; according to him, seeds sown in a soil devoid of microscopic life fail to germinate (*Compt. rend.*, 1885, **100**, 66). Owing to various technical difficulties which were subsequently realised, the observations of Duclaux could not be supported by later workers. Nilson (*J. A. C. S.*, 1904, **26**, 289) observed that lactic acid bacteria are invariably associated with germinating barley, and showed that the acid reacts with the seed proteins to produce enzymes, which convert the reserve food material into soluble forms required by the embryo. Nilson's observations were contradicted by Windisch and Schoenewold (*Woch. Brau.*, 1905, **22**, 200) who stated that barley can be completely sterilised by treatment with a solution of mercuric chloride in alcohol, without impairing the germinating capacity. Pickering (*J. Agric. Sci.*, 1907-8, **2**, 411)

observed that sterilised seeds of *Lilium italicum*, *Lilium perenne*, clover and spinach could be germinated on agar-agar jelly. Fowler and Sen found that acetone-producing organisms of the type of *B. Granulobacter pectinovorum* (E. P., 4845 of 1919) occurred in association with many cereals (*J. Indian Inst. Sci.*, 1921, 4, 119). Fowler and Sreenivasaya noticed that a bacterium is always present on the seed of *Cassia tora*, used for reducing indigo in the indigenous dye-vat, and is responsible for the reduction of indigo to the soluble leuco-compound (*J. Indian Inst. Sci.*, 1921, 4, 205). Fowler and Marsden concluded that an organism associated with coconut husk facilitates retting by loosening the fibre from the pectins and gums (*J. Indian Inst. Sci.*, 1924, 7, 39). In view of the above and other observations concerning the occurrence of specific bacteria on the indigo-seed and inside the poppy-head, Fowler and Christie decided that there is a definite symbiotic or mutual relation between seeds and some of the micro-organisms associated with them (*J. Indian Inst. Sci.*, 1924, 7, 253).

Other authors have adduced evidence that there is hereditary symbiosis between certain species of plants and some of the associated micro-organisms. Bernard observed a specific fungus to be necessary for germination of the orchid seed: the fungus was present on the growing plant and in the ripening seed (*Compt. rend.*, 1899, 128, 1253). This observation was subsequently modified by Bernard himself and later contradicted by Kundson (*Bot. Gaz.*, 1922, 73, 1) who germinated varieties of orchid in the absence of fungi. Freeman noted that *Lolium temulentum* is associated with a fungus carried from one generation to another through the seed (*Proc. Roy. Soc.*, 1902, 71, 27). Mische found that *Ardisia crispa* harbours an organism which is hereditary to the plant, but he did not succeed in separating the organism from the seed, so there is no definite proof that they exist in symbiosis (cited from *Zentralbl. Bakt.*, Abt. II, 1913, 37, 142). Faber observed a similar phenomenon in the case of many *Rubiaceae* (*Jahr. Wissen. Bot.*, 1924, 54, 243).

Symbiosis between two dissimilar forms of life would mean that they (a) are in some definite physiological association, (b) are, generally, mutually helpful and (c) cannot live or function normally without each other. A critical study of the various observations cited above will show that in no case has it been demonstrated that a specific organism or group of organisms is in symbiotic relationship with the associated seed.

Nilson's observations (*loc. cit.*) are not supported by sufficient biochemical evidence to show that the associated organism (a) penetrates the seed-coat and (b) produces lactic acid which reacts with the proteins of the embryo to form enzymes. The specific bacteria described by Fowler and some of his co-workers (*loc. cit.*) are really

those selected under the natural or artificial conditions favouring such organisms to the exclusion of most other forms of life: such observations cannot be inferred to throw any light on the relation between the original seed, or coconut, as the case may be, and the selected organism. The observation that a certain organism occurs in constant association with a seed does not necessarily mean that it is physiologically related, but indicates that the development and active functioning of both are favoured by similar natural conditions. Viewed in this light it appears doubtful whether any of the organisms claimed to be hereditary symbionts can be correctly thus described; many of them have not been isolated, so that one of the essential proofs of their symbiotic association is still lacking. Even Bernard's observations (*loc. cit.*), though of considerable interest, do not show whether the sterilised seeds were really in the right condition for germination. In the case of a thick-coated and slow-germinating seed like the orchid, proper ageing of the mature seed and prolonged steeping under favourable conditions are necessary to ensure germination; moreover, as appears from the present investigation, sufficient proof was not adduced to show that the antiseptic used for the sterilisation of the seed-coat was not the cause of suppressed germination.

In view of the inconclusive nature of the evidence so far obtained and the need for some definite information regarding the relation between seeds and micro-organisms in general, the present investigation was undertaken. The studies have been directed to ascertaining whether (*a*) there are specific organisms in definite physiological association with seeds and (*b*) micro-organisms either individually or totally have any influence, direct or indirect, on germination of the seed and the development of the seedling.

The following is an outline of the series of experiments conducted:—

(1) Systematic examination of seeds for micro-organisms occurring outside as well as inside the seed-coat. (2) Experiments to effect the sterilisation of the seed without injury to its vitality, and comparative study of various sterilising agents used for that purpose. (3) Germination of sterilised seeds under aseptic conditions as compared with that of unsterilised seeds under ordinary conditions, with special reference to time of germination and growth of seedling. (4) Response of seeds previously sterilised and then inoculated with the seed-organisms, or with different other forms of micro-flora, as compared with those of sterilised and untreated seeds.

Such seeds and micro-organisms as were found to be specifically associated with each other were tested to determine whether they satisfied the following conditions for symbiosis:—(*a*) Constant

occurrence of specific organisms in the seeds obtained from different samples. (b) If the symbiosis is absolute, the symbionts will fail to flourish without each other, i.e. the seed may fail to germinate, or the seedling may not thrive in the absence of the symbiont; similarly the symbiotic organisms would fail to grow in the absence of the seed. (c) If the symbiosis is facultative, the symbionts will flourish more vigorously when in association than they would when separated from each other.

### EXPERIMENTAL.

*Search for specific bacteria.*—Wherever possible, seeds were taken from fresh fruits under aseptic conditions in the laboratory and examined for bacteria harboured either on the seed-coat or in the body of the seeds. In cases where only seeds could be obtained, attempts were made to determine whether any particular organism was constantly associated with a number of representative specimens. The following seeds were examined:—*Citrus acida* (fresh fruit), *Ferronia elephantis* (fresh fruit), *Lycopersicon esculentum* (fresh fruit), *Arachis hypogaea* (dry pods), *Cajanus indicus* (green pods), *Cocos nucifera* (whole fresh fruit from bazaar and local gardens), *Areca catechu* (whole fresh fruit), *Ardisia humiliana* (from local Botanical Garden), *Ardisia crenulata* (from Penang, F.M.S.), *Hordeum vulgare* (bazaar variety), *Triticum sativum* (bazaar variety), *Oryza sativa* (bazaar varieties), *Trigonella fenugracum* (bazaar variety), *Pisum sativum* (green and dry pods), *Cassia tora* (bazaar variety), *Indigofera tinctoria* (from Bihar?), *Anona squamosa* (fresh fruit), *Gossypium herbaceum*, *Cannabis sativa* and *Ardisia crispa* (from Ceylon).

Before removing the seeds, the fruits were subjected to such sterilising treatment as they could tolerate without effect on the seeds. The lemon (*Citrus acida*), the tomato (*Lycopersicon esculentum*) and ground-nut (*Arachis hypogaea*) were each dipped for 20 minutes in mercuric chloride solution (2 in 1000) and then passed through flame. The fruit was then punctured with a sterilised scalpel near a gas flame, the seed taken out with sterilised forceps and placed in petri-dishes containing nutrient agar and in tubes containing nutrient broth. The contents of some tubes were subsequently covered with layers of molten or liquid paraffin to facilitate the growth of such strict as well as facultative anaerobes as may be present. Several fruits (4-6) were examined in each case and from each fruit generally five cultures were made.

In the cases of wood-apple (*Ferronia elephantis*), coconut (*Cocos nucifera*) and arecanut (*Areca catechu*) more drastic methods of sterilisation were adopted. Thus the coconut was first dipped in mercuric chloride solution (2 in 1000) for 15 minutes, and then passed through flame. Holding the nut near the flame, a triangular cut was made in

the centre of a face with a sharp, sterilised scalpel. A few shreds of husk from the deeper layer removed by sterilised forceps were then plated and tubed in the manner described above; usually, three specimens were collected from each. Tender coconuts were given similar treatment, but the period of soaking in mercuric chloride was reduced to 10 minutes.

When a seed was examined for organisms inside the seed-coat or in the body of the seed, the surface was sterilised by steeping it in mercuric chloride (2 in 1000) for 10 to 15 minutes. The seed was then washed with sterile water, crushed in a sterilised mortar and transferred immediately to a petri-dish or a broth-tube. Control experiments were also made to determine whether the outside of the seeds had been completely sterilised. In the case of seeds obtained from the market, no attempt was made to study the flora on the seed-coat because it was expected that being exposed to dust, the organisms would be essentially those of the soil. Separation of the organisms inside the seeds was effected in the manner already described, the incubations being at 37°; the dishes and tubes were examined at frequent intervals.

The observations may be summarised as follows:—From over fifty inoculations made with each seed, growths were observed in three or four cases only, and the organisms isolated were not identical, the majority being the commoner forms of soil fungi, chiefly *Penicillia* and *Aspergilli* which appeared on the plates or in the tubes at the end of the first four or five days. Some of the growths were those of bacteria, chiefly of the spore-forming and spreading type such as *B. dendroides*, and were particularly prominent in the cases of *Cocos nucifera* (coconut) and *Areca catechu* (arecanut). They were also occasionally encountered in the seeds of *Gossypium herbaceum* (cotton) which, owing to the persistent adherence of fine fibres and the film of oil around the seed-coat, could not be properly wetted even after prolonged soaking in the sterilising solution. Control experiments revealed the same organisms in small numbers on the surface of seeds or fruits treated with sterilising agents in the manner described already.

A critical study of the results showed that the stray growths observed were merely accidental and probably due more to contamination from outside than to the presence of living organisms within. The heterogeneity of the isolated flora and the identity of most with the commoner soil organisms suggest that none of the bacteria or fungi was specific to the seeds or fruits concerned, and that they were all derived from the soil which might have settled on them as dust. The slow germination of the fungi or the spreading bacteria suggests that they were originally present as spores and thus remained unaffected even after prolonged treatment with antiseptics.

Although they cast doubt on the efficacy of what have been hitherto regarded as efficient methods of sterilisation, these observations do not preclude the possible occurrence of symbiotic organisms which could not be isolated by the foregoing methods; the specific organisms might not grow on artificial nutrient media, but might develop readily on their natural hosts, namely, the seeds themselves.

*Attempts to isolate specific organisms.*—The seeds examined were wheat, barley, paddy, beans (*Phaseolus vulgaris*), peas, *Cassia tora*, indigo, methi (*Trigonella fenugroecum*), cotton, *Ardisia humiliana*, *A. crenulata* (Penang) and *A. crispa*. Fully developed and healthy specimens were chosen and after being treated in three different ways to remove the adhering foreign flora as far as possible, were inoculated into (a) sterile water and (b) sterile mashes (3 per cent.) of the different seeds. The treatment was (1) washing repeatedly with sterile water, (2) soaking in dilute copper sulphate solution (0.5 per cent.) for 3 minutes followed by washing with sterile water, and (3) treating with mercuric chloride (0.1 per cent.) for 2 minutes followed by washing with sterile water. After inoculation as described above, the tubes were incubated at 37° and examined at frequent intervals. They were also diluted with sterile saline and plated on (a) nutrient agar and (b) seed-mash agar.

The contents of all the tubes turned acid within a day or two after inoculation. The acids were identified as lactic, acetic and butyric, particularly the last, the quantities of which appeared to increase on standing. There was also noticeable evolution of gas at the end of the first few days. The tubes inoculated with seeds washed with sterile water showed the greatest activity and presented a larger variety of flora, particularly fungi, than the others. Microscopic examination did not show the preponderance of any particular organism or group of organisms in any case.

The growth on the plates confirmed the above observations. Fungi (*Penicillia* and *Aspergilli*) and acid-producers were the next prominent organisms on plates prepared from diluted washings of seeds soaked in sterile water. Fungi were generally absent and acid-producers present only in small numbers on plates prepared from suspensions containing seeds previously washed with copper sulphate or mercuric chloride. Only in three cases were seen organisms other than those mentioned above. The plates made from cotton-seed were characterised by the occurrence of bacilli growing in rapidly spreading colonies and morphologically allied to *B. dendroides*: in the case of the coconut and arecanut bacteria similar to those described by Fowler and Marsden (*loc. cit.*) were also observed. Such organisms were always present on the fibres irrespective of locality or the stage of ripeness; they were absent from the respective kernel and

also from the milk in the case of the coconut. Some preliminary experiments also showed that in addition to the organism described by Fowler and Marsden many of the other organisms isolated from coconut and arecanut fibre could ferment sterile suspension of the husk more or less vigorously, thereby bringing about partial retting.

The foregoing observations appear to cast doubt on the possible association of the different fruits or seeds with specific organisms. The prominence of a certain organism or group of organisms under given experimental conditions would only indicate that the medium into which they were inoculated is favourable to their growth. Since in none of the experiments described above, no single organism was met with to the exclusion of others, it appears doubtful whether the study of any of those organisms would throw light on the possible response of the seed to the biochemical environment indicated by their presence.

Some experiments were conducted to determine whether the isolation of the more resistant bacteria such as those described by Weizmann (*loc. cit.*) and Fowler and Sen (*loc. cit.*) from cereals indicated any specific relation between those seeds and the allied organisms. The different seeds used in the previous trials were treated in the manner described by Weizmann, and the sub-culturing of the mixed flora first obtained was repeated at the boiling temperature of the mash until the flora finally obtained consisted only of organisms possessing identical morphological characteristics. The fermenting mashes were also examined for the production of gases, acidity, acetone and other allied products.

It was noted that in all cases the surviving organisms were rod-like, spore-forming bacteria similar to those described by Weizmann and others: the products of their activity were also the same. These observations combined with those of Fowler and Subrahmanyam (*J. Indian Inst. Sci.*, 1925, 8A, 71) who found acetone-producing bacteria in soil, sewage and various forms of vegetation, show that such organisms could not be regarded as exclusive associates of seeds. In view of the fact that the micro-flora on the surface of seeds, and those occurring in sewage are primarily those of the soil, it would appear that the thermo-resistant acetone-producing bacteria are normal inhabitants of the soil and not specific associates of seeds. The foregoing observations have thus shown that the different seed-treatments for the isolation of organisms suspected to be symbionts result only in partial sterilisation of the seed-coat and the selection of a few resistant species able to withstand the treatment.

*Effect of sterilisation of seed-coat on the germination of seeds.*— Although no direct evidence could be obtained to suggest the existence of any symbiotic or mutual relation between seeds and micro-organisms, it was still considered possible that the presence of the associated

organisms, either individually or as a group, might be necessary either to accelerate germination or to help in the development of the seedling. Some experiments were therefore carried out to determine the effect of partial or entire sterilisation of seed-coat by treatment with various antiseptics. Phenol, ethyl alcohol, formaldehyde, bleaching powder, copper sulphate and mercuric chloride were tried for the purpose and it was found that the last three were efficient, and at the same time the most convenient.

The seed-bed was made up of fine white sand prepared by boiling coarse sand with concentrated hydrochloric acid and then washing repeatedly to remove the last traces of acid. The sand was collected in glass basins with close fitting covers and conical beakers fitted with cotton wool plugs, and after being moistened, autoclaved intermittently at 10 lbs. and for 20 minutes until inoculation tests showed that the sand was completely free from living organisms.

Some preliminary tests were made on paddy, wheat and barley to determine the extent of sterilisation attained after treatment with dilute solutions of copper sulphate (1-2 per cent.) and mercuric chloride (0.1-0.2 per cent.), respectively. The grains in lots of 10 to 15 were treated with the antiseptic solutions for 5 minutes in each case. Representative specimens of the treated grains were shaken with sterile saline and plated out on nutrient agar in the usual way. On examining the plates on the fourth day it was found that the 0.2 per cent. solution of mercuric chloride had almost completely sterilised the seed-coats while solutions of lower strength were not so effective. Treatment with copper sulphate was still less satisfactory.

Following Wilson's method (*Amer. J. of Bot.*, 1915, 2, 420) bleaching powder was tried for a similar purpose. From a commercial specimen of bleaching powder containing 25 per cent. available chlorine, aqueous solutions containing 2, 4 and 6 per cent. of available chlorine were prepared. The grains were treated as before and tested for the extent of the sterility of their seed-coats. It was observed that short-period treatments were not very effective and that soaking for about six hours in solutions containing 4 or 6 per cent. available chlorine was necessary completely to sterilise the seed-coats. It was however noted that the seeds thus treated did not germinate properly; the percentage of germination was low and the development of such seedlings as emerged was very poor. Treatment for even 8 hours with the solution containing 2 per cent. available chlorine was not effective in sterilising the seeds. In view of the weak sterilising action of copper sulphate and bleaching powder, and the possible unfavourable effect of prolonged treatment with such antiseptics, it was considered desirable to use mercuric chloride in subsequent experiments.

To determine the direct action of mercuric chloride adhering to the seeds in minute quantity representative specimens of the different grains were soaked in solutions of concentration ranging from 0.0025 to 0.2 per cent. for 10 minutes in each case, and allowed to germinate in sand-basins prepared as described. The percentage of germination, the general appearance of the seedling, and the development of shoot and root were determined in each case.

The observations showed that although all the seeds germinated at the end of three days, none of the seedlings developed properly. Those from seeds previously soaked in concentrations of 0.0025-0.05 per cent. suffered the least, but even they showed distinct weakness in root development within the first six days. After a week all the seedlings showed the effect of mercury poisoning in increasing degree. The experiments were therefore repeated washing the treated seeds with sterile water and comparing the growth of seedlings raised from them with those from untreated seeds. The observations are recorded in Table I.

TABLE I.

Concentration of mercuric chloride	Grain treated and washed	Grain treated and not washed
	<i>Paddy</i>	
1/1000	Seedling identical in appearance and growth with control	Roots not properly developed
2/1000	Apparently healthy roots; somewhat long	Roots short ; seedling unhealthy
	<i>Barley</i>	
1/1000	Identical with control	Roots slightly shorter than those of the control
2/1000	Resembles control ; roots not well developed	Poor root development
	<i>Wheat</i>	
1/1000	Similar to control ; bright in colour	Weak and unhealthy roots
2/1000	Root system elongated ; otherwise the same as control	Do.

The observations show that partial sterilisation of the seed-coat with a powerful antiseptic does not appreciably affect germination and the development of the seedling if the last traces of antiseptic are carefully removed. The above experiment was then repeated with

peas and tomato; the antiseptic was not so effective as with the cereals, but the germination results were similar to those described above.

It may be inferred from the foregoing observations that sterilisation of the seed-coat does not affect either the germination of the seed or the growth of the seedling if the last traces of antiseptic are removed. The seedling is highly sensitive to chemical poisons, and as in the case of copper sulphate or bleaching powder would show the effect thereof even if the seed-coat is only partially sterilised. It is not improbable that some of the earlier observations attributed to absence of associated micro-organisms were really due to minute quantities of antiseptic not completely removed after sterilising the seed-coat.

Further experiments were then made with the seeds of *Cassia tora*, *Trigonella fenugræcum*, *Phaseolus vulgaris* (French beans), tomato, peas, barley, wheat and paddy to ascertain whether addition of the mixed flora associated with the different seeds or soil extract to the sterilised seeds had any beneficial effect on the development of the respective seedlings. The seeds were sterilised by treatment with a 0.1 per cent. solution of mercuric chloride, and washed with sterile water to remove the antiseptic. They were then divided into three lots of which one was control, one treated with washings from the respective seeds and the third with suspensions of soil extract. The seeds were then spread out to germinate on sand-beds prepared in the manner described already. The rate of germination of the seed as well as the development of the seedling were determined in all the cases.

The results showed that the variations of treatment produced no visible effect on the germination of the different seeds: nor did the seedlings show any difference in the cases of paddy, *Cassia tora*, *Trigonella fenugræcum*, *Phaseolus vulgaris*, and peas. Some of the seedlings of barley, wheat and tomato appeared to be slightly benefited by suspensions of the mixed flora present on their respective seed-coats, but the observation could not be confirmed on repetition. The seedlings whether mixed with the associated flora or not, developed in a similar manner in all the cases. It was, therefore, inferred that (a) the associated bacteria have no influence on either germination or the development of the seedling, and (b) the better formation of seedlings observed in some cases was merely accidental and probably due more to the presence of certain readily available nutrients in the soil or seed extract than to the presence of micro-organisms.

*Effect of inoculation of specific organisms on germination and growth of seedlings.*—Having seen that no direct evidence of any

mutual relation between the seeds and associated micro-flora could be obtained, some attempts were made to determine whether active cultures of some of the commoner types of soil-organisms could appreciably influence germination.

Nilson (*loc. cit.*) observed that during the germination of barley, the lactic acid bacteria then prominent pass inside the hull of the grain. The previous observations of the present authors not having supported such a view, some experiments were conducted to test the above by more direct methods.

The seeds of *Dolichos lablab*, peas, barley, wheat and paddy were steeped in water, salt solution (1 per cent.), sulphuric acid (0.001 *N*) and sodium carbonate (0.5 per cent.) respectively for varying lengths of time (30 minutes–6 hours) to soften the seed-coats, and then spread out on sand-beds (unsterilised) to germinate. Representative specimens were removed at convenient intervals, fresh sections prepared, fixed, stained with methylene blue and examined for possible penetration by micro-organisms, but in no case could any living organism be observed.

In view of the possibility of the penetrating organisms not being present in sufficient numbers, some further experiments were made by adding aqueous suspensions of representative soil-organisms to various seeds soaked in different ways as described. The organisms used for inoculation were, *B. coli*, *B. lactis aerogenes*, *Hansenia apiculata*, a wild yeast originally found associated with rice, *Aspergillus niger*, and *Actinomyces Scabies*. The seeds were inoculated by soaking them momentarily in aqueous suspensions of the different organisms, and then spread out on sand to germinate. Representative specimens were removed at convenient intervals, washed with alcohol and sectioned; the sections were fixed, stained and examined for penetration.

It was observed that although the organisms persisted on the outer coats they had not penetrated the seed, even in a single instance. The seeds germinated at about the same rate and in the same proportion as those untreated. The growth of seedlings also appeared to be unaffected by the inoculated organisms. A few seedlings that had been moistened too freely were partly overgrown by the vegetative mycelia of *Aspergillus niger*, but were otherwise unaffected. Since however such fungus growths appear occasionally even on normally germinating seeds, if wetted profusely, it may be inferred that the overgrowth of the fungus was merely accidental and caused primarily by faulty watering. It follows that micro-organisms do not penetrate the seed and cannot therefore directly influence germination. The foregoing observations do not, however, preclude the possibility of products formed by the activity of the inoculated organisms passing

into the seed and thereby affecting, indirectly, the development of the seedling.

*Is the presence of micro-organisms necessary for the production of lactic acid in the germinating seed?*—Nilson (*loc. cit.*) showed that lactic acid is present inside the germinating seed. In view of the previous observations it appeared probable that minute quantities of acid produced outside the seed-coat by different organisms might have passed into the seed and thus helped the germination. Some experiments were therefore made to determine whether lactic acid is present in germinating seeds and, if so, whether micro-organisms are necessary to produce it.

Healthy seeds of paddy, wheat, barley and peas were selected and one half sown on a sand-bed, the other half being sterilised with mercuric chloride as described already and then sown. Representative specimens of the seeds were taken at different stages before and after germination and examined for the presence of lactic acid. The wet seeds were ground to paste, treated with dilute sulphuric acid, dried on the water-bath and extracted in four soxhlets with ether. After distilling off the ether, the extracts were taken up with water and tested for lactic acid.

It was observed that all the seeds whether sterilised or not, gave positive Uffelmann and thiophene tests for lactic acid at different stages in the course of their germination. In the case of wheat the acid was found to be formed within 8 hours after sowing, while in the case of barley and paddy the minimum periods required for acid formation were 30 and 60 hours, respectively; the reaction was less pronounced with peas. The small amount of acid precluded quantitative determinations being made by any of the known methods.

It may be inferred from the above that (*a*) lactic acid is formed in minute quantities in all germinating seeds and (*b*) the acid is produced by the seed itself and not by micro-organisms inside the seed-coat. The function of lactic acid in the germinating seed has not received much attention; it is probable that, as in the case of higher organisms, it is a product of carbohydrate metabolism and associated with respiration.

*Effect of chemical treatment on germination and the activity of associated micro-organisms.*—Although the previous observations had shown that the seed and the associated micro-flora are physiologically independent, it still appeared probable that they might exist in mutual relation under certain abnormal conditions. To test the above, the seeds of *Dolichos lablab*, paddy, barley and wheat were sown in sand-beds and treated with (*a*) antiseptics (thymol and phenol), (*b*) a bacterial food (glucose), (*c*) a product of bacterial metabolism (lactic acid), (*a*) a plant nutrient (Knop's food solution), (*e*) an inert salt (sodium

chloride) and (f) distilled water (control). Finely powdered thymol (0.05 per cent.) mixed uniformly with the sand; phenol (0.05 and 0.025 per cent.), lactic acid and glucose (0.05 per cent.) were used in aqueous solution.

Knop's food solution was prepared by dissolving 0.25 g. of magnesium sulphate, 1 g. of calcium nitrate, 0.15 g. of dihydrogen potassium phosphate, 0.12 g. of potassium chloride and a trace of ferric chloride in 1 litre of water, and added in quantity sufficient to moisten the seeds; sodium chloride was added in a 0.05 per cent. solution. The moisture content of the sand-bed was maintained constant by adding distilled water from day to day. The root-length and shoot-height of seedlings as also the numbers of bacteria adhering to seeds were determined at the end of six days. The observations have been summarised in Tables IIa, IIb, IIc and II d.

TABLE IIa.

PADDY

Seed treated with	Average root length in mm.	Average shoot length in mm.	Bacteria per seed (average) in thousands	Remarks
Distilled water	26.7	55.3	73	Seedlings normal (control)
Thymol	No germination	...	18	No visible effect on the seed-coat
Sodium chloride	22.7	48.3	59	Seedlings healthy, but not well developed
Glucose	23.7	49.0	Uncountably large. Profuse growth of fungi.	Seedlings healthy, but somewhat pale
Lactic acid	Poor. Over-grown with fungi	Poor. Over-grown with fungi	Uncountably large. Profuse growth of fungi	Seedlings started healthy—growth checked after 3 days—Warped root development
Knop's nutrient	34.0	77.3	97	Seedlings bright and healthy—rapid development
Phenol (0.05 per cent.)	No germination	...	7	Seed-coat turned dark brown

TABLE IIb.

## BARLEY

Seed treated with	Average root length in mm.	Average shoot length in mm.	Bacteria per seed (average) in thousands	Remarks
Distilled water	40.0	71.0	71	Development of seedling normal (control)
Thymol	No germination	...	21	No visible effect on seed-coat
Sodium chloride	35.3	61.7	64	Seedlings normal
Glucose	33.7	57.3	Uncountable. Spreading colonies	Seedlings not well developed
Lactic acid	12.7 Partly overgrown with fungi	36.3 Partly overgrown with fungi	Uncountable. Overgrown with fungi	Growth of seedlings suppressed after the first few days—root development poor
Knop's nutrient	50.6	100.0	115 Largely overgrown with fungi	Seedlings healthy and well formed
Phenol (0.05 per cent.)	No germination	...	11	Seed-coat stained brown

TABLE IIc.

## WHEAT

Seed treated with	Average root length in mm.	Average shoot length in mm.	Bacteria per seed (average) in thousands	Remarks
Distilled water	40.0	58.3	69	Seedlings normal (control)
Thymol	No germination	...	14	No germination even after 12 days
Sodium chloride	38.0	59.0	54	Seedlings healthy—indistinguishable from control

TABLE IIc.—(contd.)

## WHEAT

Seed treated with	Average root length in mm.	Average shoot length in mm.	Bacteria per seed (average) in thousands	Remarks
Glucose	32.3	49.0	Uncountable. Plates overgrown with fungi	Seedlings unhealthy
Lactic acid	9.0	31.0	Overgrown with fungi	Seedlings developed normally during the first 4 days; roots warped after that date
Knop's nutrient	45.0	87.0	84	Seedlings healthy and well developed
Phenol (0.05 per cent.)	No germination	...	7	Seed-coat stained black

TABLE II d.

*Dolichos lablab*

Seed treated with	Average root length in mm.	Average shoot length in mm.	Bacteria per seed (average) in thousands	Remarks
Distilled water	42.7	75.6	85	Seedlings normal (control)
Thymol	No germination	...	23	Only one seed germinated
Sodium chloride	42.7	63.0	69	Seedlings apparently healthy
Glucose	41.3	63.3	Uncountable. Profuse growth of fungi	Seedlings pale and unhealthy
Lactic acid	Overgrown with fungi	Overgrown with fungi	Profuse growth of fungi	After the shoot was about 20 mm. in length growth ceased

TABLE II d.—(contd.)

*Dolichos lablab*

Seed treated with	Average root length in mm.	Average shoot length in mm.	Bacteria per seed (average) in thousands	Remarks
Knop's nutrient	64.7	100.7	77	Seedlings healthy and well developed
Phenol (0.05 per cent.)	No germination	...	11	Most of the seeds blackened

It was observed also that the roots of seedlings raised on lactic acid tended to turn away from the seed-bed, thereby indicating that the acid was toxic. The roots tended to turn upward rather than go deeper into the sand-bed, so that the seedlings showed signs of being starved for want of sufficient nutrition. Profuse growths of fungi (chiefly *Penicillia*) were noticeable around seeds treated with glucose and lactic acid. Seeds treated with Knop's nutrient were generally clean and free from visible growths of fungi though the plates prepared from them contained numerous colonies of fungi in addition to those of the bacteria. Seedlings raised on glucose were invariably overgrown with fungi which covered the roots as well as the shoots and thus interfered with the normal development of the seedlings. Thymol had less inhibiting action on the bacteria than phenol, but was equally effective in checking the germination of the seed. Owing perhaps to the low concentration, sodium chloride was without much effect on either seedlings or bacteria: from the present results, its action would appear to be one of slight depression on the growth of seedlings as well as the multiplication of bacteria.

The foregoing observations indicate that seeds and associated micro-organisms do not always respond in the same manner to chemical treatment. In presence of a readily decomposable carbohydrate like glucose, bacteria and fungi increase at rapid rates while the growth of the seedling is actually suppressed. Lactic acid is toxic to the seedling, but encourages the spread of fungi. Knop's solution is beneficial to the seedlings but the associated micro-organisms do not respond so readily to it as they do to lactic acid or glucose; there is marked increase in bacterial numbers in that nutrient, but the growth is very much less than in glucose. Being essentially saprophytes, these organisms are naturally more favoured by a nutrient containing readily available carbon than by one which is made up of only minerals. The micro-organism numbers are slightly reduced and the development of seedlings appreciably retarded by

treatment with sodium chloride. Phenol and thymol are both toxic to bacteria and inhibit germination. Since similar toxic effects have been noted in the case of the micro-organisms even when they occur independently of the seeds, it may be inferred that the above observations cannot be attributed to any mutual relation between those two forms of life.

To determine whether the abnormal effects noticed in the case of seedlings treated with lactic acid were due to the high concentration, some experiments in treating the seeds of *Dolichos lablab*, paddy, wheat and barley with lactic acid solutions of lower concentrations (0.0025, 0.001 and 0.0005 per cent.) were made, germination and development of the seedlings being studied in the manner described already. It was observed that although the seeds germinated readily, the growth of the seedlings was rapidly affected as in the previous experiment. Root development was greatly retarded and, after the first four days, growth was almost negligible. The effect was most pronounced in the case of seeds treated with 0.0025 per cent. solution of the acid.

Some experiments were also conducted to determine whether the inhibitive action of thymol on germination was due to its chemical nature, or to its germicidal action in checking bacterial growth on the germination beds. The seeds were sown on beds treated with thymol-water (water filtered from finely powdered crystals) and the moisture content maintained by sprinkling the beds with the same extract once in twenty-four hours. Rates of germination as well as bacterial counts were determined in each case. It was observed that all the grains on the thymol-water beds germinated in the normal way, thereby indicating that low concentrations of that antiseptic were harmless. The bacterial counts on the other hand were about the same as in the previous experiment. This observation lends further support to the inference that germination is unconnected with bacterial activity.

Similar experiments with seeds treated with toluenated water showed that although the bacterial numbers were not appreciably affected, the seeds did not germinate at all. Addition of seed or soil extracts to the treated seeds did not lead to any improvement, thereby showing that toluene is deadly to the embryo and its action is not influenced by the presence or absence of micro-organisms. This observation also shows that the seeds and the associated bacteria are really independent of each other and respond differently to the same treatment.

#### DISCUSSION.

The results of the present investigation do not support the existence of any symbiotic, mutual, or other physiological relation between seeds and associated micro-organisms. The two forms of life,

one dormant and the other active, occur only in physical association and, under normal conditions, function independently of each other. They do generally respond in the same manner to a variety of physical and physico-chemical conditions, but do not, on that account, develop any physiological association with each other. Under some conditions, one form may be favoured almost to the suppression of the other; under others, the seed and associated bacteria may be both destroyed.

The earlier part of the work has shown that the normal micro-flora of the seed are essentially those derived from the soil. The dust which covers the seed-coat is only finely divided soil and, naturally, contains all the commoner soil-flora. All attempts to isolate the hypothetical specific associates have resulted only in selection of one or more of soil-organisms as could withstand the treatment given. If the seed-mash is fermented by the associated organisms, first lactic and then butyric acid bacteria increase considerably: the fungi normally present as spores and which, in consequence, germinate rather slowly, multiply rapidly at this stage and soon suppress all the other forms of life. The above are distinctly cases of natural selection. The bacteria mentioned above produce acids and gases which discourage most other forms of life. The fungi thrive best in slightly acid media and are favoured by the presence of organic acids, which they readily oxidise to carbon dioxide: so they multiply rapidly after the first few days and in doing so, suppress all the other micro-organisms. In a like manner, treatment with antiseptics to isolate the specific associates of seeds also results only in the selection of the more resistant organisms. Non-sporers are readily killed while those which occur as spores are only partially destroyed by the antiseptic. Spores of fungi and certain soil bacteria persist even after prolonged treatment with the commoner antiseptics and are thus the only organisms to be isolated after the treatment. The bacteria and the fungi thus found in association with the seed cannot be described as being in symbiotic relation with the latter. They are only artificial selections under the conditions of the experiments and are no more symbiotic with the seeds than those organisms which were killed by the antiseptics. Nor can such organisms be described as being specific because the flora isolated after treatment with antiseptics were not consistent and included a large number of bacteria and fungi.

No evidence was obtained to indicate the presence of specific micro-organisms inside any germinating seeds. Such bacteria and fungi as were isolated from seeds of which the outer coats had been previously sterilised by heat and antiseptics were (*a*) generally some of the commoner soil forms and (*b*) not consistent in composition. This observation in conjunction with the previous one to the effect that the seed-coat is generally only partially sterilised as the result of the treatments mentioned above leads to the inference that the

organisms isolated by such methods were essentially those which were left on the seed-coats and which had originally been derived from the soil.

A similar argument could be applied to show that the thermo-resistant, Fitz-type organisms isolated from seed-mashes are (*a*) only artificial selections and (*b*) not specific to any of the seeds as they are actually derived from the soil. It is no doubt possible that there are several varieties of such organisms occurring under identical conditions and that some are physiologically more active than others: it is also possible that the biochemical activity of such organisms and their efficiency in converting starchy materials into acetone, butyl alcohol and other allied products are largely determined by the chemical composition of the seed or the tuber with which they are associated and on which they are raised. But the fact remains that they are normal inhabitants of the soil and bear no specific relationship to any of the seeds or tubers from which they are isolated.

A certain amount of evidence in favour of symbiosis between seeds and associated bacteria has been adduced by some of the earlier workers from their studies on the germination of seeds of which the outer coats were sterilised by heat or antiseptics. Such investigations did not however include tests to determine whether (*a*) the sterilisation of the seed-coat was normally complete, and (*b*) the treatments adopted for sterilisation did not have any direct effect on germination. The present studies have shown that none of the commoner methods of sterilisation leads to complete elimination of all the associated micro-organisms. The germinating seed is left with a few resistant forms which in the absence of the other micro-flora necessary to maintain the biological equilibrium may adversely affect the seedling. Moreover, the seed-coat always retains traces of the antiseptic used for the sterilisation: such chemicals being highly toxic to the seedlings, the growth of the latter is rapidly affected. Thus traces of bleaching powder, copper sulphate, mercuric chloride, formaldehyde, or toluene though without much effect on the associated micro-organisms, are either deadly to the embryo or highly poisonous to the seedlings. The effects persist after adding suspensions of the mixed soil-flora, or sufficient soil extract to restore the original conditions. Thus it would be seen that the effects attributed to the removal of associated bacteria were really due to the toxic action of antiseptics used for sterilising the seed-coats. Further proof of this fact was obtained from the observation that the treated seeds from which the last traces of the antiseptic were carefully removed, germinated just as satisfactorily as those from which the associated micro-organisms were not removed.

Addition of specific organisms or products of microbial activity did not help either germination or the development of the seedling.

In fact, some of them proved to be inimical to the growth of the seedling. The presence of minute quantities of lactic acid in all germinating seeds, even after sterilisation of the seed-coat shows that the acid is a normal product of seed-metabolism and is unconnected with the presence of micro-organisms.

Treatment with bacterial or plant nutrients, or chemical poisons do not generally evoke similar response from the seeds and the associated micro-organisms; conditions favouring one form of life may be distinctly inimical to the other. The same series of experiments also showed that even when the response was found to be similar, the seeds and the associated bacteria behaved in the same way as they would have done if they had occurred independently of each other.

It may perhaps be argued that the nitrogen-fixers associated with the root nodules of *Leguminosæ* are specific symbionts of those seeds. Recent work has however shown that such is not the case. The nodule organisms are no doubt present in the soil in which the legume has been established for some years, or into which the organisms have been introduced by inoculation. The bacteria may even occur in large numbers on the seed-coats: but they do not bring about germination or help in the early nourishment of the seedling. They move towards the roots and form the nodules only after the seedlings have established themselves. It therefore follows that the nodule bacteria are in active symbiotic association with the growing plants and not with the seeds.

The results of the present investigation show conclusively that under normal conditions, the seeds and the associated micro-organisms are physiologically unrelated to each other. The occurrence of the latter on seed-coats is merely due to the inevitable accident of finely divided soil being scattered by wind as dust. The prominence of a single organism or group of organisms under certain artificial conditions would be merely cases of selection and thus bear no relation to the natural association of such forms with the seed.

#### SUMMARY.

1. A study of the nature and the distribution of micro-organisms associated with a large number and variety of seeds showed that the former are all derived from the soil and that none of them is specific to any of the seeds.
2. Treatment with heat or antiseptics does not sterilise the seed-coat completely, but leads to the selection of one or more resistant forms, which are not generally specific to the seeds from which they are isolated.

3. There is no direct evidence to show that living organisms are present inside healthy seeds.

4. Sterilisation of the seed-coat does not in any way affect either germination or development of the seedling, provided the last traces of the antiseptic used for the purpose are removed.

5. Addition of active cultures of specific organisms does not help germination, but may prove inimical to the growth of the seedling under certain adverse conditions.

6. The presence of micro-organisms, within or without the seed, is not necessary for the production of lactic acid which is formed in minute quantities inside all germinating seeds.

7. Treatment with nutrients, stimulants or toxics does not generally evoke a similar response from seeds and associated bacteria.

8. Under natural conditions, seeds and soil micro-organisms are inevitable associates which are physiologically unrelated and live independently of each other.

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