QUEENSLAND DEPARTMENT OF PRIMARY INDUSTRIES
DIVISION OF PLANT INDUSTRY BULLETIN No. 554

RELATIONSHIP OF SOIL MOISTURE, TEMPERATURE AND ALKALINITY TO A SOYBEAN NODULATION FAILURE

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SUMMARY

The roles of low soil moisture, high soil temperature and soil alkalinity in a soybean nodulation failure in an alkaline black soil were assessed.

Desiccation of nodule bacteria in seed inocula was unlikely in soil moist enough to germinate seed.

Temperatures in the seed zone differed by as much as 9°C between wet and dry soil in midsummer, 32°C being the maximum recorded in wet soil. Exposing peat-inoculated seed to 40°C for 8 hr did not seriously affect nodulation but 50 and 60° for 4 hr did.

The optimum pH for growth of *R. japonicum* strains was pH 6·0, with very little growth at pH 8·5 or pH 9·0. Strains of *R. meliloti*, *R. leguminosarum* and *R. japonicum* (serotype 135) showed a higher tolerance to alkalinity in survival tests than did other random strains of *R. japonicum*. Seed pelleting with acidic materials gave better nodulation than with alkaline materials, lime having a suppressing effect.

It is concluded that soil temperatures, although not the main cause, could lead to nodulation failure when coupled with soil alkalinity influences. *Rhizobium* strain selection in the field, higher inoculum levels and seed pelleting were the most promising approaches to its solution.

I. INTRODUCTION

Suitable rhizobia for soybean (*Glycine max*) are often lacking in south-eastern Queensland soils. Nodulation difficulties from inoculated soybeans on alkaline clay soils of the Darling Downs, Queensland, have been reported by Harty (1964) and Diatloff (1967). Preliminary studies in black earth soils (pH 8·0-8·4) have narrowed the problem to one of survival of the seed inoculum. Nitrate inhibition had previously been discounted (Diatloff 1967) as well as seed coat toxicity, soil nutrient deficiency and microbial antagonism in the soil (Diatloff, unpublished data). Three other possible causes of nodulation failure have been postulated, namely, damage from high soil temperatures, desiccation from low soil moisture, and soil alkalinity.

This paper describes investigations into these three factors.

II. MATERIALS, METHODS AND RESULTS

(a) General Experimental Data

Bacterial strains and cultures.—Twenty-three strains of *Rhizobium* were used; those with the prefix QA were isolated from plants growing in black soil while those with a CB prefix were obtained through Dr. D. O. Norris, C.S.I.R.O. Division of Tropical Pastures, Brisbane. Cultures were isolated and maintained on yeast-mannitol agar. Peat cultures were prepared by incorporating liquid cultures on yeast-mannitol broth into fine autoclaved peat and were stored at 5°C.

Soils.—The black earths on which these investigations were carried out are the "black" self-mulching clay soils of the open plains and lower hill slopes of the Darling Downs (Beckmann and Thompson 1960). Most occur as a gilgai complex, so the profile of the depression differs from that of the small puff where a tongue of lighter coloured subsoil containing soft carbonate rises to the surface. With cultivation the 0-6 in. horizon becomes more or less homogeneous, with a distinctly alkaline reaction (pH 7·4-8·4). The three experimental sites were at Hermitage (pH 7·4), Jondaryan (pH 8·3) and Bongeen (pH 8·2).

Growth and survival studies.—Growth studies were carried out in broth cultures by following colorimetrically the change in turbidity after 10 days' growth. Nodule bacteria were grown in duplicate tubes of yeast-mannitol broth (Norris 1964). Survival studies were carried out in duplicate tubes of mineral salts solution (Arnon and Hoagland 1940). The change in bacterial numbers after 4 days was followed by the drop-plate count method.

Field trials.—Trials were carried out in midsummer. The inoculated seeds were planted 1½-2 in. deep in single-row plots 20 ft long and 6 ft apart. Three replications were used in randomized blocks and an uninoculated control was included in all cases. From each replication 30-50 plants were removed by digging and the roots washed before examining for nodulation. The number of nodulated plants was recorded as well as nodule number, and position on the root system was noted.

(b) High Soil Temperature

Occurrence of high soil temperatures.—With current soybean varieties, December plantings are most productive (Anon. 1964, p. 12). The frequency distribution of daily maximum soil temperature for a 6-monthly period (October 24, 1963-April 14, 1964) was compiled from data obtained by J. K. Leslie (personal communication) from thermocouple readings in a bare fallow at Jondaryan (Figure 1). While a diminishing temperature gradient occurred away from the soil surface, a temperature of 40°C was exceeded at a depth of 3 in. on 13 days. Bowen and Kennedy (1959) considered this temperature critical to the survival of some rhizobia.

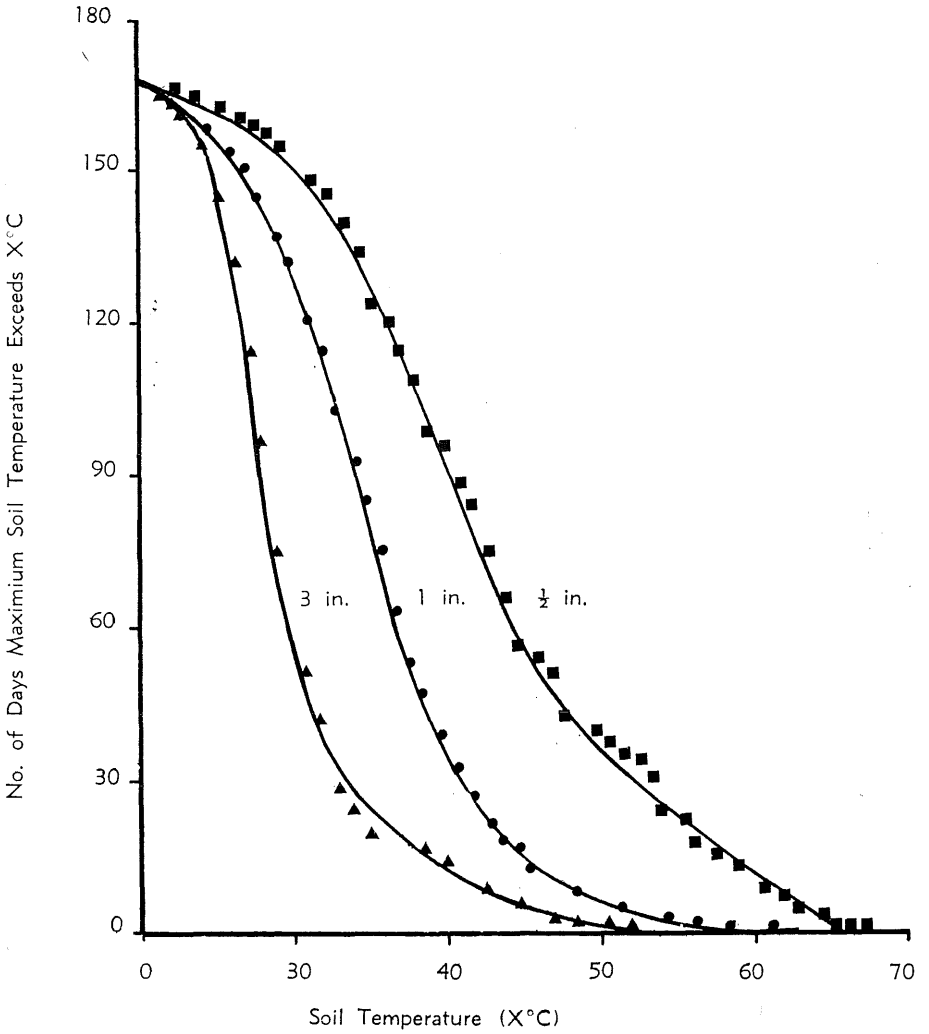

Fig. 1.—Cumulative frequency distribution of maximum soil temperature at three depths in black soil.

Since the soil moisture is known to modify soil temperatures (Dravid 1940), recordings were made at Hermitage in both wet and dry soil. January 25, 1968, was taken as a typical clear, hot summer day. Soil temperatures were recorded by distance thermograph in two plots, one of which had been irrigated 6 days earlier while the other was left untreated. The moisture levels at 0-6 in. depth were 36 and 27% respectively, the permanent wilting point of the soil being approximately 26% (J. K. Leslie, personal communication). The probes were inserted at a depth of 1½-2 in. in each plot. Figure 2 shows the recorded soil and ambient air temperatures for a 14 hr period and their duration.

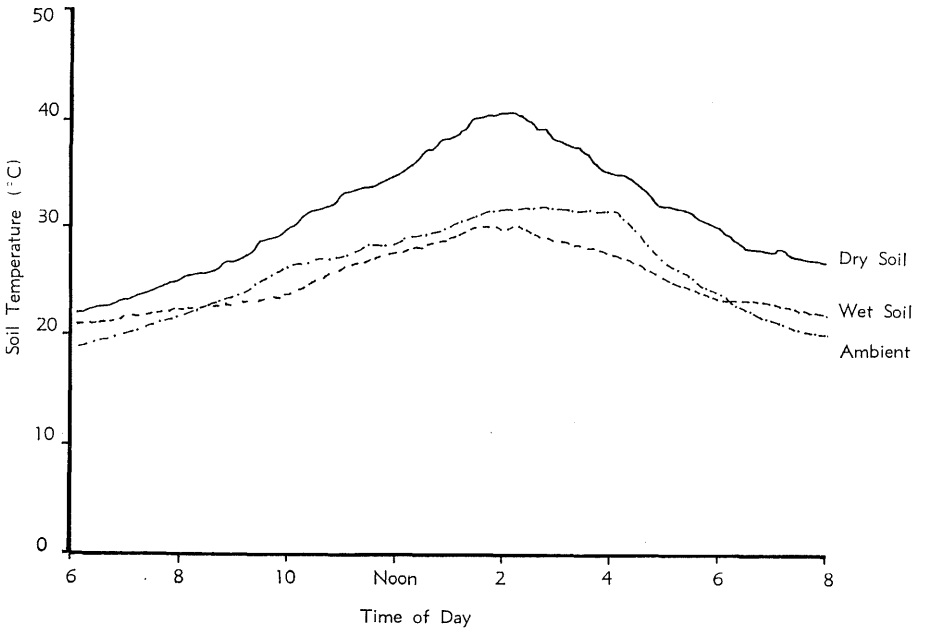

Fig. 2.—Temperature occurring in wet and dry soil in midsummer.

The temperatures recorded in moist soil were as much as 9°C lower than those in dry soil. The maximum daily temperature in moist soil was 32°C while in dry soil it was 41°C. The latter temperature occurred for a period of approximately 1 hr, although 35°C was exceeded for a period of 3½ hr.

Experiment 1: Effect on Nodulation of High-temperature Treatment of Inoculated Seed

Inoculated soybean seeds were sown into 2 in. terracotta pots of moist unsterile soil (32·4% moisture). The seeds were inoculated with a peat culture of strain CB1003 to give a count of $4{\cdot}3 \times 10^4$ rhizobia per seed. Two seeds were planted in each pot, which had been brought up to the experimental temperature prior to planting and covered with a petri dish. To avoid contamination during handling, each pot was wrapped in sterile paper. The pots were then subjected to temperature treatment at 30, 40, 50 and 60°C for 4, 8, 24, 96 and 168 hr in an incubator. One series of pots was held at 30°C for 4 days until germination, followed by 50°C for 4 hr so as to expose a developing rhizosphere population to high-temperature treatment. Ten replications were used. At the predetermined time intervals pots were removed from the incubator, the paper cover was removed and a sterile cotton wick was inserted through the hole in the bottom. The pot was then placed into the neck of a 2 lb glass jar partly filled with sterile deionized water. Plants were then grown for 4 weeks in a constant-temperature growth cabinet at 26°C. Due to poor emergence in one-third of the pots, an assessment was based on only one plant. Pots with plants showing any nodulation were classed as positive (Figure 3).

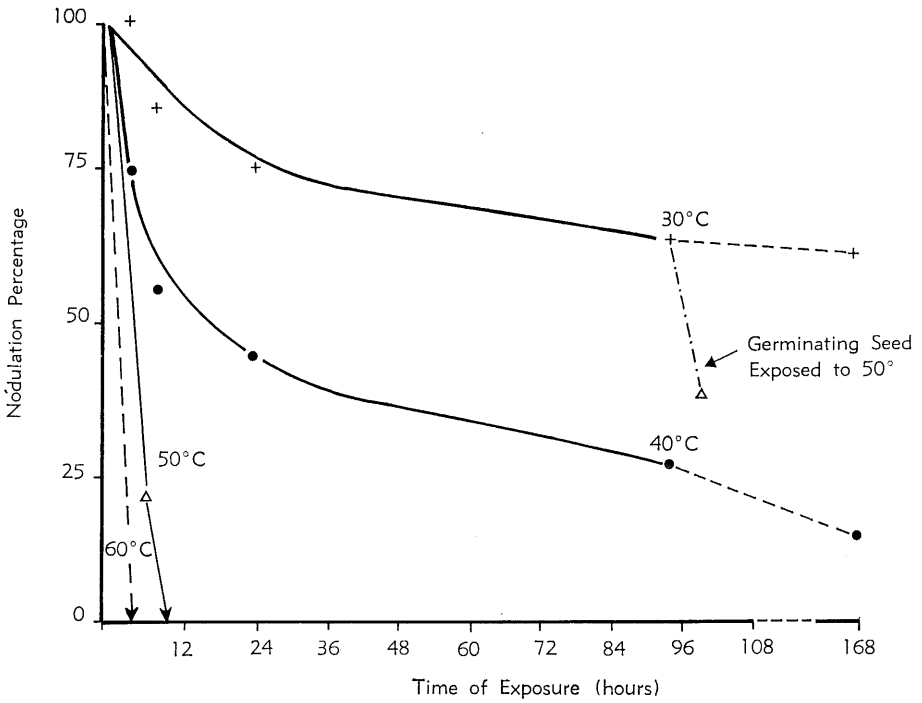

Fig. 3.—Nodulation of soybeans following high-temperature exposure of inoculated seed.

Exposure of inoculated seed to 50 and 60°C was particularly damaging to subsequent nodulation even for the shorter period of 4 hr. Exposure to 40° did not appear to be critical up to 8 hr, but beyond this period nodulation was noticeably reduced. Exposure of germinating seed to 50° for 4 hr after being held at 30° for 4 days also reduced nodulation, but the rhizosphere bacteria did not appear to be more sensitive to heat treatment than did bacteria in the peat inoculant.

(c) Low Soil Moisture

Experiment 1: Relation between Soil Moisture and Relative Humidity of the Soil Atmosphere

Rhizobium is particularly susceptible to death on desiccation following seed inoculation. Vincent, Thompson and Donovan (1962) found *R. trifolii* to be susceptible at both high (60% R.H.) and low humidity (0% R.H.), although there was some multiplication at 100% R.H. Since under normal planting conditions bacteria are unlikely to come in contact with free water, the nature of the soil atmosphere surrounding the inoculated seed is important in determining the fate of the inoculum.

To establish the relationship between the humidity of the soil atmosphere and soil moisture in black soil, 12 humidity chambers were set up with relative humidities ranging from R.H. 4·7% to R.H. 100% by the use of different concentrations of sulphuric acid (Solomon 1951). The chambers consisted of

1 lb honey jars containing 100 ml of acid above which two small soil samples (approx. 15 g) in cotton gauze were suspended. The soil and humidities were allowed to equilibrate for 3 weeks at 30°C, after which time no weight changes occurred. The moisture content of the soil was determined after drying at 105°C overnight.

The relative humidity bore a sigmoid relationship to soil moisture (Figure 4), which had to fall below permanent wilting point of black soil (26% moisture) before the R.H. of the soil atmosphere fell below 100%.

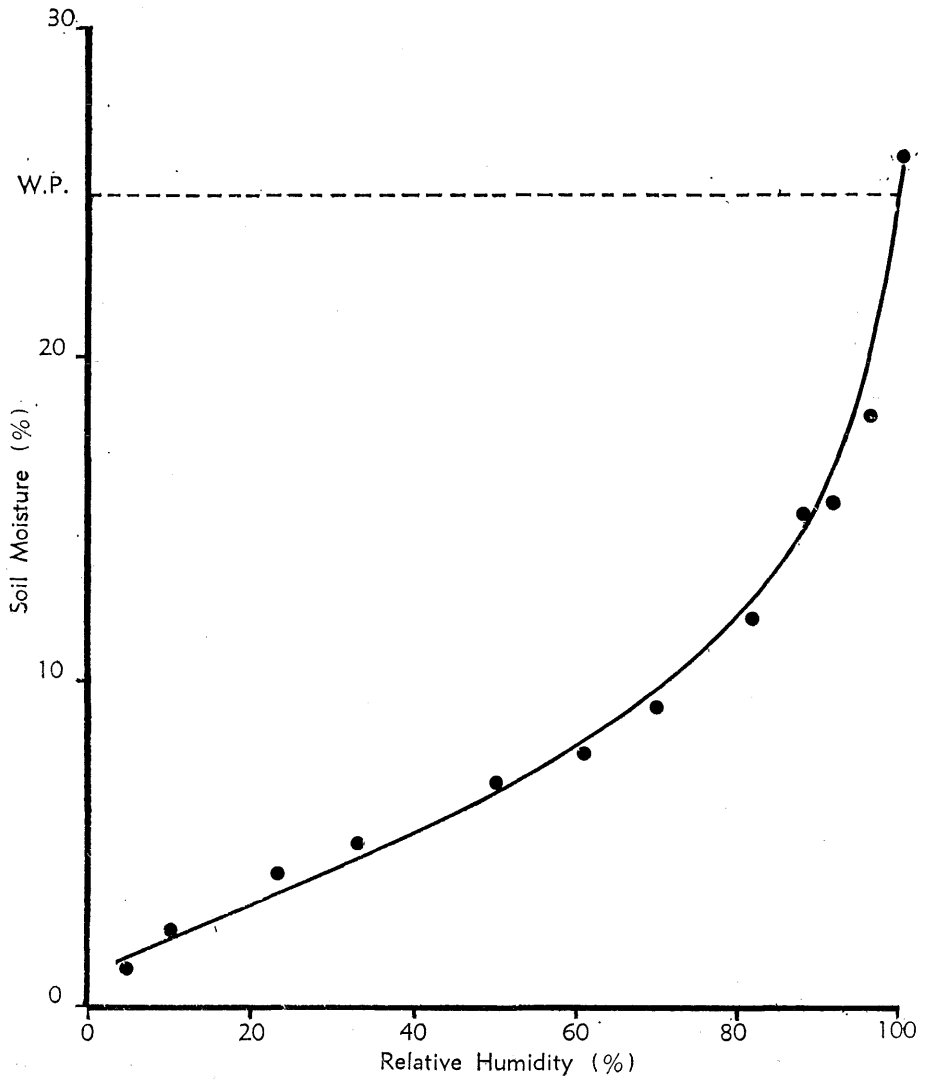

Fig. 4.—Relation between soil moisture and relative humidity in the soil atmosphere at 30°C.

(d) Soil Alkalinity

Experiment 1: Effect of pH on the Growth of *R. japonicum*

Seventeen strains of *R. japonicum* were grown in the yeast-mannitol broth with adjusted pH ranging from pH 5·5 to 9·0. Little growth occurred at pH 8·5 or pH 9·0 (Figure 5), but growth was favourable at pH 5·5. The optimum pH for growth was 6·0.

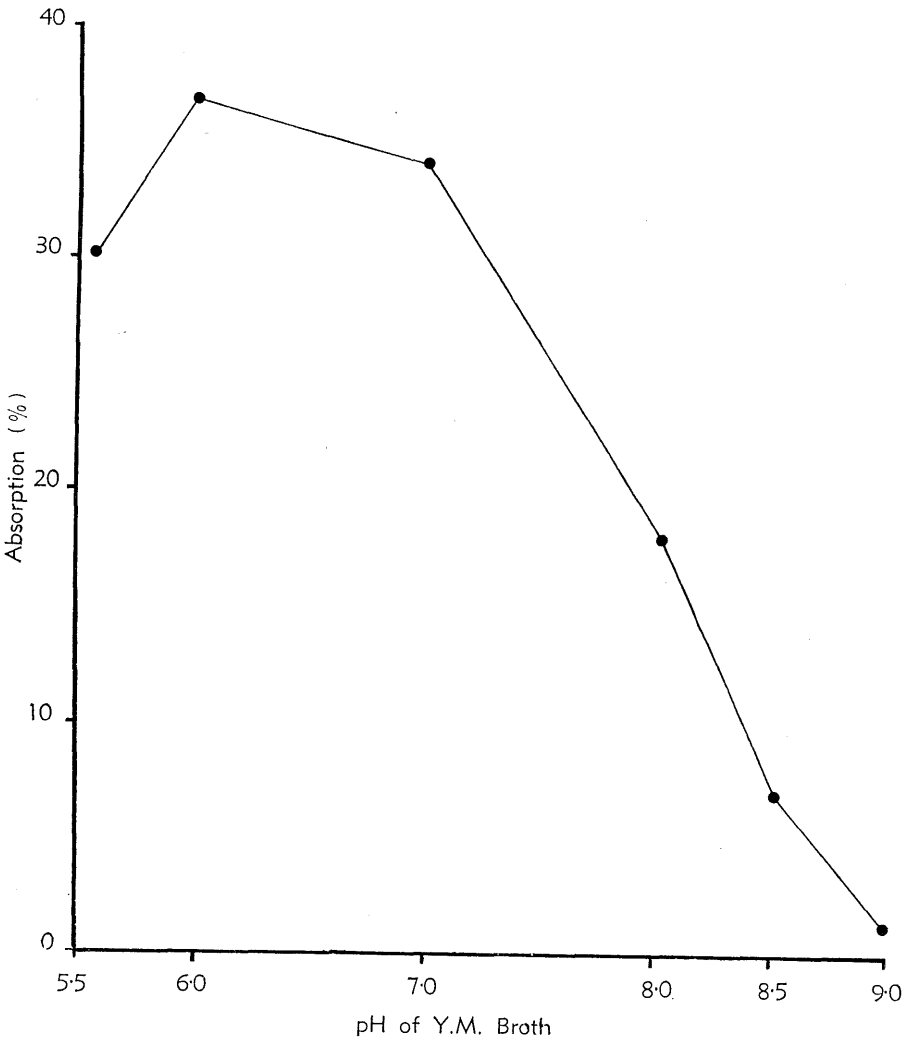

Fig. 5.—Effect of pH on the growth of *Rhizobium japonicum* at 10 days. (Mean of 17 strains.)

Experiment 2: Effect of pH on Survival in Mineral Salts Solution

The 16 strains listed in Table 1 were tested over the pH range 3·8, 5·2, 6·4, 9·0 and 9·5.

TABLE 1

COMPARATIVE SURVIVAL OF *Rhizobium* SPP. IN MINERAL-SALTS SOLUTIONS AT TWO pH LEVELS

Strain	Log decline/day at pH 3·8 / Log decline/day at pH 9·5 $\left(\frac{k3{\cdot}8}{k9{\cdot}5}\right)$
Group I (soybean)—random selection of strains	
QA891 a	1·043
QA981	0·943
CB1003	0·883
QA1009	0·816
CB1795	0·888
CB1809	0·862
Mean	0·905
Group II (soybean)—alkali-tolerant strains of serotype 135†	
CB2021	1·048
CB2022	1·397
CB2024	1·087
CB2025	0·939
Mean	1·118
Group III—isolated from legumes growing in black soils	
QA1039 (*Medicago*)	1·060
QA1040 (*Vicia*)	0·996
QA1041 (*Melilotus*)	1·071
QA1042 (*Psoralea*)	1·000
QA1038 (*Vigna*)	1·134*
Mean	1·031
Group IV	
CB376 (*Lotononis*)	0·898

* $\frac{k3{\cdot}8}{k9{\cdot}0}$ † Damirgi, Frederick and Anderson 1967

Drop plate counts were made immediately after setting up the experiment and 4 days later. The results were computed as log decline / day k. The ratio $\frac{k3{\cdot}8}{k9{\cdot}5}$ was selected to express the relative acid or alkali tolerance of the strains (Table 1). Values approaching or in excess of 1·0 show some alkali tolerance.

There were differing tolerances to various pH levels between species of *Rhizobium* and between strains of *R. japonicum*. The four groups of strains showed the following mean ratios:

Group I	0·905
Group II	1·118
Group III	1·031
Group IV	0·898

Group II and III strains showed some alkali tolerance, while the randomly selected soybean strains (except for QA891a) were not alkali-tolerant. The isolate from *Lotononis* (a legume well adapted to acid soils) had a low k value for all pH levels.

Experiment 3: Effect of Seed Pelleting of Different pH Reaction and Inoculum Levels on Nodulation

The various pelleting techniques, inoculum levels and types of cultures were compared in this trial as listed in Table 2. A sticker consisting of 10% w/v maltose and 20% w/v gum arabic was used to form pellets, while 10% w/v maltose was added to broth cultures. Seeds were inoculated 15 hr before planting

into a moist soil at Bongeen (pH 8·2) in November 1964. Identical seed, soil and inoculants were checked in a glasshouse planting. Prompt germination occurred in the field and growth continued under favourable moisture conditions. A maximum temperature of 45°C was recorded in the seed zone within the first 10 days. The trial was harvested after 10 weeks and examined for nodulation. At the same time, plant samples from some of the treatments were removed from the field for rhizosphere studies. The plants were pulled from the soft earth and shaken lightly. The lateral roots were removed with sterile scissors and washed in sterile water. Nodules were avoided where present. Soybean seedlings in bottle-jar units were then inoculated with these washings, using one jar of three plants per original plant and assessing nodulation after 4 weeks.

TABLE 2

EFFECT OF LEVEL OF INOCULUM AND PELLETING ON THE NODULATION OF SOYBEAN IN THE FIELD AND GLASSHOUSE

Treatment	Percentage Nodulated Plants	
	Field	Glasshouse
Heavy peat inoculation (2·7 x 10^7 rhizobia per seed) ..	50	100
Low peat inoculation (2·0 x 10^5 rhizobia per seed)	18	66
Low peat + carbon pellet	0·7	100
Low peat + lime pellet	0	66
YM bacterial broth (1·8 x 10^5 rhizobia per seed)	1·6	100
Uninoculated control	0	0

The nodulation problem in this instance was peculiar to the field in that all glasshouse treatments produced 66–100% nodulated plants (Table 2). Neither activated carbon nor lime pelleting afforded the applied inoculum any advantage over simple inoculation and both were in fact deleterious (Table 2). Commercial calcium phosphate pelleting (pH 5·2) severely reduced seed germination and no worthwhile results were obtained. The peat-inoculated seed led to better nodulation than did broth-inoculated seed, while a higher inoculum level in peat also improved nodulation. A correlation between nodulation and survival of rhizobia in the rhizosphere was established (Table 3). There was no recovery of rhizobia from non-nodulated plants.

TABLE 3

NODULATION OF SOYBEAN SEEDLINGS INOCULATED WITH RHIZOSPHERE WASHINGS FROM FIELD SOYBEAN PLANTS

Source of Rhizosphere Washing	No. of Test Units Showing Nodulation
Nodulated plants from heavy peat inoculation treatment	6/6
Un-nodulated plants from heavy peat inoculation treatment	1/9
Un-nodulated plants from low peat inoculation treatment	0/9
Un-nodulated plants from low peat inoculation treatment with lime pellet ..	0/9
Un-nodulated plants from YM bacterial broth treatment	1/7

Experiment 4: Seed Pelleting with Materials Less Alkaline than Lime

Five materials were selected, their pH being determined in distilled water when the slurry was at paste consistency. These are shown in Table 4. Two stickers, 5% Cellofas (pH 7·0) and 45% gum arabic (pH 5·3), were used. A peat culture of strain CB1809 containing 5·5 x 10^8 rhizobia per g was applied

at commercial rates to soybean seed (cv. Leslie) and the seed pelleted with the various materials. Two treatments without pellets were also included—an inoculated control (Cellofas and gum arabic with peat inoculum) and an uninoculated control (Cellofas and gum arabic with sterile peat). The 14 treatments were planted at two sites in December 1967. Nodulation was assessed after 6 weeks.

TABLE 4

PERCENTAGE NODULATION OF SOYBEANS WITH VARIOUS SEED PELLETING AND STICKER TREATMENTS AT HERMITAGE AND JONDARYAN

Treatment and Pelleting Material	Gum Arabic Sticker		Cellofas Sticker	
	Jondaryan	Hermitage	Jondaryan	Hermitage
Sterile peat	0	7·06 (0·26)*	0	4·30 (0·20)*
Peat inoculum	33·87 (0·62)*	39·33 (0·67)	21·81 (0·48)*	43·12 (0·71)
Peat inoculum + Ca phosphate/gypsum 2 : 1 pellet, pH 6·4	50·03 (0·78)	55·87 (0·84)	30·03 (0·58)	39·61 (0·68)
Peat inoculum + Ca phosphate/gypsum 1 : 2 pellet, pH 6·8	45·26 (0·73)	75·77 (1·05)	33·98 (0·62)	65·57 (0·94)
Peat inoculum + black soil pellet, pH 7·5	19·79 (0·46)	40·56 (0·69)	18·41 (0·44)	20·35 (0·46)
Peat inoculum + gypsum pellet, pH 8·0	33·36 (0·61)	48·50 (0·77)	24·81 (0·52)	60·31 (0·88)
Peat inoculum + lime pellet, pH 8·3	0	5·83 (0·24)	0	18·47 (0·44)
L.S.D. 5%	(0·10)	(0·21)	(0·10)	(0·21)
L.S.D. 1%	(0·14)	(0·28)	(0·14)	(0·28)

* Arcsine $\sqrt{\text{per cent}}$

No growth differences occurred, presumably because of moderately high soil nitrate levels. Again, the lime pelleting depressed nodulation in both sites and with both stickers, the effect being more marked on the more alkaline soil of Jondaryan. Gypsum improved nodulation at Hermitage only, while the calcium phosphate mixtures stimulated nodulation in five out of eight instances (Table 4). Gum arabic was consistently better than Cellofas at Jondaryan only. At both sites there appeared to be a trend in relationship between nodulation and the pH of the pelleting materials (Hermitage, $r = -0{\cdot}78$; Jondaryan $r = -0{\cdot}58$). *P* was greater but approaching 0·05 significance on both sites.

Experiment 5: *Rhizobium* Strain Performance in Alkaline Soils

Nine glasshouse-tested *Rhizobium* strains, including four alkali-tolerant strains of serotype 135, were tested in the field at two sites. The design incorporated 11 treatments on three varieties (Table 5). The viable count of the peat cultures used for inoculating seeds exceeded $3{\cdot}5 \times 10^7$ rhizobia / g peat in all cases. The plantings were made in December 1967 and January 1968 and both areas were harvested after 6 weeks' growth. The soils were moist throughout the growing period and the highest daily maximum soil temperature at 2 in. was 35°C at Hermitage and 37°C at Jondaryan. Nodulation was assessed on 40–50 plants dug from each plot and examined for crown and lateral nodulation and nodule count.

TABLE 5

PERCENTAGE NODULATED PLANTS IN FOUR VARIETIES OF SOYBEANS BY NINE STRAINS OF *R. japonicum* AT HERMITAGE AND JONDARYAN

Treatment	Hermitage	Jondaryan
ECR 973 CONGO		
CB2025	65·81 (0·94)*	51·00 (0·79)*
CB2024	16·71 (0·42)	28·41 (0·56)
CB2022	26·85 (0·54)	45·50 (0·74)
CB2021	13·81 (0·38)	22·48 (0·49)
CB1809	76·06 (1·06)	56·35 (0·84)
CB1003	13·13 (0·37)	11·26 (0·34)
QA1009	33·17 (0·61)	54·64 (0·83)
QA981	84·94 (1·17)	88·48 (1·22)
QA891a	83·24 (1·14)	96·53 (1·38)
Uninoculated control ..	0	0
LESLIE		
CB2025	34·77 (0·63)	34·63 (0·62)
CB2024	14·23 (0·38)	10·12 (0·32)
CB2022	24·24 (0·51)	39·01 (0·67)
CB2021	9·68 (0·31)	9·03 (0·30)
CB1809	91·07 (1·26)	81·28 (1·12)
CB1003	7·41 (0·27)	0·42 (0·06)
QA1009	37·59 (0·66)	61·13 (0·89)
QA981	89·41 (1·23)	75·39 (1·05)
QA891a	74·98 (1·04)	74·86 (1·04)
Uninoculated control ..	0	6·0 (0·24)
HILL		
CB2025	33·82 (0·62)	
CB2024	7·94 (0·28)	
CB2022	34·72 (0·63)	
CB2021	6·29 (0·25)	
CB1809	85·97 (1·18)	
CB1003	3·80 (0·19)	
QA1009	45·78 (0·74)	
QA981	90·08 (1·25)	
QA891a	88·84 (1·23)	
Uninoculated control ..	0	
SEMSTAR		
CB2025		25·48 (0·52)
CB2024		24·95 (0·52)
CB2022		36·82 (0·65)
CB2021		12·96 (0·36)
CB1003		3·06 (0·17)
CB1809		75·71 (1·05)
QA1009		75·07 (1·04)
QA981		79·65 (1·10)
QA891a		77·18 (1·07)
Uninoculated control ..		2·14 (0·14)
L.S.D. 5%	(0·13)*	(0·22)*
L.S.D. 1%	(0·17)	(0·30)

* Arcsine $\sqrt{\text{per cent}}$

The strains differed in their infectivity both between sites and between varieties when assessed as percentage nodulation and nodule number per plant (Table 5 and Figure 6). Strains CB2021, CB2024 and CB1003 performed consistently poorly on both sites and on all varieties, while in contrast QA891a,

QA981 and CB1809 performed consistently well. As a whole, the serotype 135 strains performed worse than the other randomly selected strains (mean 26·2% *v.* 60·1%).

Strains that nodulated fewer plants also tended to produce fewer nodules per plant and a high percentage of crown nodules. There was a positive correlation between percentage nodulation and nodule number and a negative correlation between percentage crown nodulation and nodule number (Figure 6). Except for one instance at Jondaryan these correlations were significant ($P<0·01$).

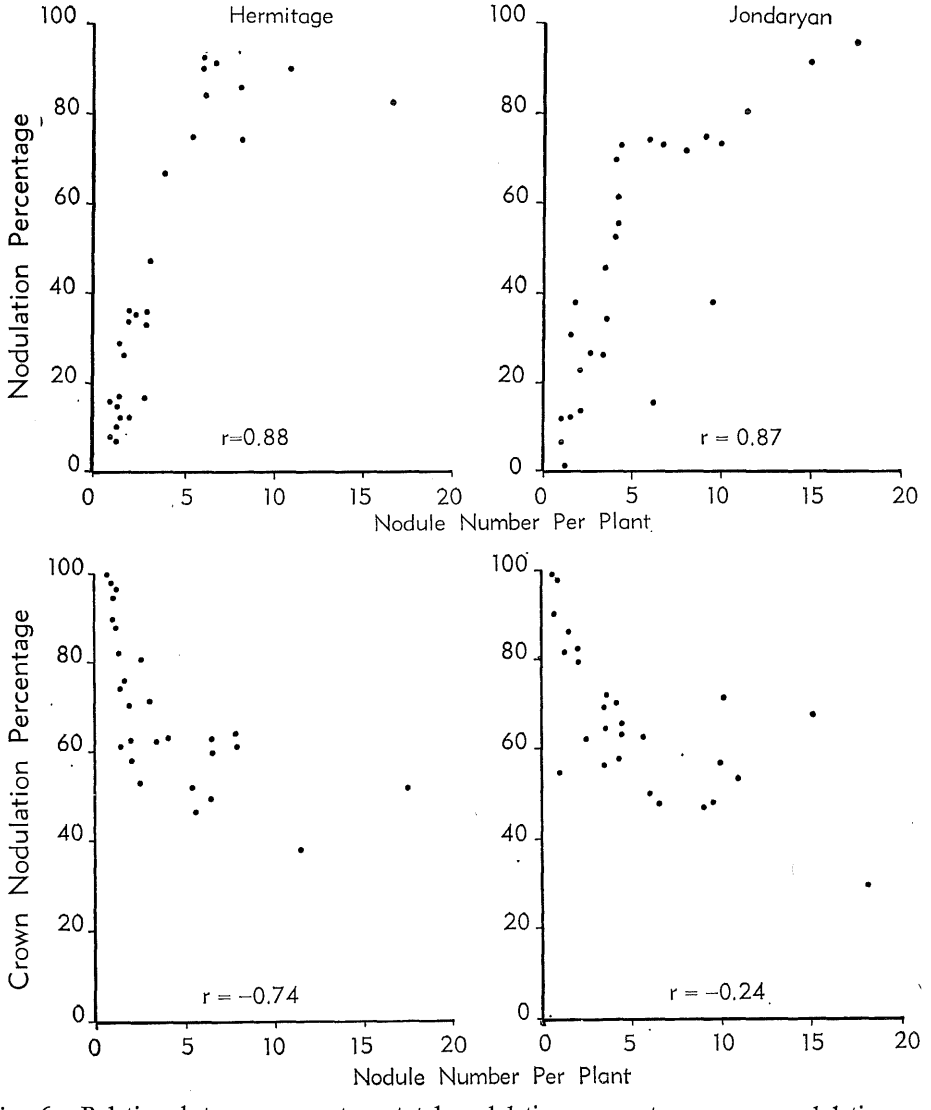

Fig. 6.—Relation between percentage total nodulation, percentage crown nodulation and nodule number with inoculated soybeans growing in black soils.

III. GENERAL DISCUSSION

The nodulation failure in soybean was apparently correlated with the failure of the seed inoculum to establish in the rhizosphere. Evidently nodule bacteria did not survive in the rhizosphere without forming nodules. Of the numerous factors so far considered, only soil temperature and soil alkalinity appear to be significant. Desiccation of the inoculum appears unlikely in soil moist enough to germinate seed.

The laboratory studies on the effects of high pH on growth and survival of soybean nodule bacteria seem to offer an explanation for the poor colonization of the rhizosphere in that neither growth nor survival is favoured by alkalinity. The black soils *per se* were not highly lethal to soybean rhizobia; however, their alkalinity might restrict the colonization of the rhizosphere coming under its influence. This is supported in the pelleting experiments by a trend towards lesser nodulation with increasing pH of the materials used. Seed pelleting experiments by Parker and Oakley (1965) and Norris (1967) indicate some lime sensitivity in other slow-growing rhizobia following planting of pelleted seed.

The poor field performance of the alkali-tolerant strains (Damirgi, Frederick and Anderson 1967), although seemingly inconsistent with the above hypothesis, could have arisen from *Rhizobium* host incompatibility for reasons quite unrelated to soil reaction.

The correlations between the number of nodulated plants, nodule position and count suggest that poorly nodulating strains had difficulty in colonizing the extending roots, although in the glasshouse all strains were capable of prolific nodulation in Leonard jars (pH 6·5). As a particular example, the strain CB1003, which was a promising strain on the acidic coastal soils (Bryan and Sharpe 1965) failed to produce anything but a few crown nodules on a small proportion of plants on alkaline black soils. This and several other reported instances of disappointing field performances of promising glasshouse selections (Cloonan and Vincent 1967; Dudman and Brockwell 1968) highlight the need for field testing of strains of *Rhizobium*.

Although high soil temperatures were recorded in a field site, these are more likely to occur in dry than in moist soil, so in the seed zone 40°C for 4 hr is unlikely to be exceeded in moist black soil. Some decline in nodulation was evident following exposure of inoculated seed to such temperature conditions, although they were by no means critical to subsequent nodulation. In terms of bacterial survival Bowen and Kennedy (1959), Chowdhury, Marshall and Parker (1968) and Wilkins (1967) infer that in moist soil or sand such a temperature regime could reduce bacterial numbers but not entirely eliminate rhizobia. Furthermore, Brockwell and Phillips (1965) have shown rhizobia in peat cultures to be remarkably resistant to high temperatures. The present field nodulation failures cannot therefore be explained by high-temperature damage alone. However, if high-temperature effects are superimposed on an unfavourable rhizosphere pH, a nodulation failure becomes more likely. Repeated high-temperature exposure on consecutive days, as might occur in the field, would help accentuate such an effect. This could well have been the case in the first seed pelleting experiment, where a maximum temperature of 45°C was recorded in the seed zone and where abnormally high bacterial numbers were required to bring about nodulation.

Although no single postulated cause adequately explains the many results obtained, the investigation has opened several promising leads to the solution of the problem. Even with an unsatisfactory strain such as CB1003, higher inoculum levels appear to overcome many of the difficulties. *Rhizobium* strain selection for ability to perform in the field appears promising but the best of the tested strains still failed to give 100% nodulation. Unfortunately, the more virulent strain QA891a is a relatively ineffective strain and has little to commend it apart from its infective nature. The fully effective strain CB1809 performed well above average on the four varieties and two sites in which it was tested, justifying its use in commercial inoculants. Pelleting with gypsum and calcium phosphate-gypsum mixtures improved nodulation, although neither of the stickers used (Cellofas and gum arabic) excelled consistently.

IV. ACKNOWLEDGEMENTS

Thanks are due to Mr. H. H. Tod and to the staff of Hermitage Research Station, Department of Primary Industries, for making cultivated land available for experimentation; to Dr. D. O. Norris, C.S.I.R.O. Cunningham Laboratory, for providing *Rhizobium* cultures and for helpful advice; and to the staff of Biometry Branch, Department of Primary Industries, for statistical analyses.

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(Received for publication May 18, 1970)

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