THE NODULATION OF ISOLATED LEGUMINOUS ROOTS[1]

Miguel Raggio,[2] Nora Raggio[2] and John G. Torrey

THE PROCESS whereby bacteria of the genus *Rhizobium* produce nodules on the roots of leguminous plants involves a complex physiological system which is poorly understood. One obvious way to simplify the system is to study the nodulation of isolated roots. Several advantages come from the use of root culture methods: the nutrition of excised roots grown in vitro can be controlled, substances normally contributed by the shoot can be excluded, and substances whose effects are to be tested can be added to the medium in known amounts.

To date only three reports have appeared describing attempts to obtain nodulation of isolated leguminous roots. Lewis and McCoy (1933) reported the first and only successful attempt. They observed the formation of 4 nodules on one root of *Phaseolus vulgaris* L. out of 60 roots tested. The poor nodulation obtained by these workers and the complete failure of others (Seppilli et al., 1941; McGonagle, 1944) made it seem particularly worth while to reinvestigate nodule formation on isolated roots.

The following facts, pertinent to the problem of the nodulation of isolated roots, had already been established for light-grown and etiolated black wax bean and soybean seedlings bearing only the cotyledons (Raggio and Raggio, 1956a). Such seedlings will nodulate when grown in a nitrate-free inorganic medium. The presence in the medium of nitrate or sucrose, alone or in combination and at the levels usually used in excised root culture, inhibits nodule formation. In all nutrient media containing sucrose, and especially in those containing nitrate in addition, great bacterial multiplication occurs.

On the basis of these conclusions the following inferences were made regarding nodulation of isolated roots: (1) Critical substances for growth and development of the primary root are known to migrate from the cotyledons into the root during the first few days of germination (McAlister and Krober, 1951). Since it has been found that the materials contained in the seeds of both black wax bean and soybean are necessary but are also sufficient for nodulation, primary roots excised from seeds germinated for several days should nodulate if adequate conditions for maintenance of growth are provided. (2) Since both nitrate and sucrose, alone or in combination, are inhibitory to nodule formation in seedlings, they may be expected to exert the same effect on excised roots, which require both substances for growth in vitro. (3) It would be expected that inoculation with rhizobia of isolated roots growing in media containing sucrose would result in an inordinate proliferation of the bacteria with a smothering effect on the roots, especially if nitrate were also present.

Using the information derived from the seedling studies and guided by the inferences listed above, an investigation of conditions leading to nodule formation on isolated roots of the above mentioned species was undertaken. A recently developed method of excised root culture (Raggio and Raggio, 1956b) which permits circumvention of the expected inhibition of nodulation by nitrate and sucrose was used.

MATERIALS AND METHODS.—Excised roots were obtained from seeds of *Phaseolus vulgaris* L., var. 'Pencil Pod' black wax bean[3] and *Glycine soja* (L.) Sieb. et Zucc., var. 'Biloxi.'[4] Seeds were surface sterilized by immersion for 10 min. in a 10 per cent solution of Saniclor (a commercial product containing 5.25 per cent sodium hypochlorite by weight). The seeds were then washed 6 times with sterile distilled water. They were next transferred aseptically to 11-cm. Petri dishes containing 20 ml. of a 1.5 per cent agar solution. Ten bean or 6 soybean seeds were sown per dish and allowed to germinate in the dark at room temperature. The germination medium was varied in early experiments, but water agar was finally used as a standard procedure.

Some preliminary experiments showed that the establishment of clones of isolated black wax bean roots when grown in modified White's medium by any of the techniques tried was not feasible. The rate of growth was slow in the first transfer. Therefore, in all experiments the root tips were obtained directly from germinated seeds. The same procedure was followed for soybean roots.

Sterile techniques were used throughout and so far as could be observed, the media were free from microörganisms other than the rhizobia with which the cultures were inoculated.

The strains of *Rhizobium phaseoli* Dangeard used were numbers 3I6C11, 3I6C13 and 3I6C17[5] (hereafter referred to as 11, 13 and 17), which

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[2] Present address: Gaspar Campos 841, Vicente López, FNGBM, Argentina.

[3] Obtained from Associated Seed Growers, Inc., Oakland, California.

[4] Obtained from Dr. H. A. Borthwick, U. S. Dept. of Agriculture, Beltsville, Maryland.

[5] Obtained from the Nitragin Co., Inc. Milwaukee, Wisconsin through the courtesy of Dr. J. C. Burton, Director of Research.

were cultured in the dark at room temperature on slants of Waksman's medium 79 (Fred and Waksman, 1928) in which 0.5 g./l. powdered Difco yeast extract replaced yeast water. The strains were transferred at 20-day intervals. On the basis of preliminary trials, strain 17 was used in most experiments.

The strain of *Rhizobium japonicum* (Kirchner) Buchanan used was 3I1b59[5] (hereafter referred to as 59), cultured in the dark at room temperature on slants of medium "AO." This medium was developed from the findings of Neal and Walker (1935) and Albrecht and McCalla (1937) and had the following composition in g./l.: 0.5 K_2HPO_4; 0.2 $MgSO_4 \cdot 7H_2O$; 0.2 NaCl; 1.5 calcium gluconate; 10 l(+)arabinose; 0.5 powdered Difco yeast extract; 15 Difco Bacto agar; glass distilled water to make one liter. Final pH of the medium was 6.8. The strain of *R. japonicum* grew more slowly than strains of *R. phaseoli* and thus transfers of the former were carried out at monthly intervals.

Bacterial inoculations were made with 4-day-old cultures of *R. phaseoli* and 6-day-old cultures of *R. japonicum*. The average surface area of a slant was 3 cm.^{2} and the amount of medium comprising each slant was 8 ml. The entire colony formed on the slant was transferred with a loop to an Erlenmeyer flask containing sterile distilled water, where a suspension was made (5 ml. distilled water per slant). Two ml. of this suspension were poured in each root culture dish.

Excised roots were grown by two different methods. In early experiments, they were grown on agar media in Petri dishes (Bonner and Addicott, 1937). Later, the method of Raggio and Raggio (1956b) was used. Essentially, it consisted of growing the excised root in an inorganic agar medium contained in an 11-cm. Petri dish, with the organic moiety of the medium provided through the base of the root from an agar medium in a small vial placed inside the dish. The media used in these studies are shown in table 1. White's medium (White, 1943) was the basic medium and was modified in various ways to suit the purposes of the experiments. The pH of the medium was measured with a model H2 Beckman glass electrode pH meter by immersing the electrodes in the liquid or the solid medium at room temperature.

All glassware was washed with detergent, rinsed repeatedly in tap and distilled water and finally air dried. Empty glassware was sterilized in a dry oven at 150°C., for 2 hr. Liquid and agar media were steam autoclaved at 15 lb./in.^{2} for 15 min. Vermiculite, sand and soil were autoclaved at the same pressure for 3 hr.

Measurements of root length were made with a flexible millimeter rule from outside the vessel. The maximum error was roughly 5 per cent of the actual length of the root.

Nodules were counted 25 days after inoculation. All nodules reported were not less than 0.5 mm. in diameter and generally were considerably larger. Nodules of this size were unmistakably different from emergent lateral roots in being rounded and cream to pink in color. Furthermore, to confirm the diagnosis, several nodules from each treatment were fixed and prepared for histological examination. In the tables, nodule numbers are presented separately as nodules formed on lateral roots and on the main root axis.

The vermiculite used was obtained from the California Zonolite Co., San Francisco, California. The sand used was Ottawa silica sand No. 24 (coarse grain), supplied by Clemco, Berkeley, California. It was washed with HCl (specific gravity 1.19) until the washings were colorless and was then thoroughly rinsed with distilled water before using.

EXPERIMENTAL RESULTS.—*The nodulation of isolated roots of black wax bean.*—All the previous attempts to obtain nodules on excised roots reported in the literature (Lewis and McCoy, 1933; Seppilli et al., 1941; McGonagle, 1944) have been carried out by the Petri dish method of isolated root culture on agar. This technique, in which the excised root lies on the surface of an agar medium, has proved very useful in studying the factors which permit growth and development of excised roots in a number of different species.

Lewis and McCoy (1933) obtained 1.7 per cent nodulation with excised black wax bean roots. A repetition of their single successful experiment under conditions as comparable as possible was thought desirable.

(a) *Experiments with the usual Petri dish method of root culture.*—Following the procedure of Lewis and McCoy (1933), black wax bean seeds were germinated for 2 days on Waksman's medium 79 and for 2 additional days in pint-size Mason jars containing 250 ml. of "Wilson's" medium (containing 0.5 per cent sucrose, cf. Wilson, 1931). After 4 days, roots ranging in initial length from 20–70 mm. were excised, the seeds removed and the roots immediately inoculated with a mixture of rhizobial strains 11, 13 and 17. The roots were continued on "Wilson's" medium for 25 days in the dark at 26°C. in a high humidity room. The results presented in table 2 (Exp. No. 7) show that only 5 roots out of 192 (or 2.6 per cent) of the inoculated roots formed nodules. This result is in very good agreement with that obtained by Lewis and McCoy (1933).

In a similar experiment (Exp. No. 22, table 2), seeds were germinated on 1.5 per cent water agar for 4 days, when the whole roots were excised and transferred to Mason jars containing 100 ml. of medium "O." The roots were inoculated with strain 17 immediately and placed in the dark at 26°C. in a high humidity room. Of 143 roots thus treated, 12 roots (8 per cent) nodulated.

In the next experiment (Exp. No. 20), bacterial

TABLE 1. *Composition of the nutrient media used for the cultivation of isolated roots for studies of nodulation (mg./l.)*

Substances	Designation of medium: "Plus nitrate"	"Nitrate free"	"O"
Inorganic constituents:			
$Ca(NO_3)_2 \cdot 4H_2O$	287.1	—	—
KNO_3	80.0	—	—
$CaCO_3$	—	—	3000.0
$CaCl_2 \cdot 2H_2O$	—	—	300.0
$CaSO_4 \cdot 2H_2O$	—	206.5	200.0
KCl	65.0	124.6	65.0
KH_2PO_4	—	—	200.0
$MgSO_4 \cdot 7H_2O$	736.8	736.8	700.0
$NaH_2PO_4 \cdot H_2O$	19.0	19.0	—
$Na_2SO_4 \cdot 10H_2O$	453.1	453.1	450.0
KI	.75	.75	.75
$FeCl_3$	1.5	1.5	1.5
Microelements: H_3BO_3, 1.5; $MnSO_4 \cdot H_2O$, 4.5; $Na_2MoO_4 \cdot 2H_2O$, 0.25; $CuSO_4 \cdot 5H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 1.5.	+	+	+
Organic constituents:			
Glycine	3.0	3.0	3.0
Vitamins: nicotinic acid, 0.5; pyridoxine, 0.1; thiamin-HCl, 0.1.	+	+	+
Sucrose	20 g.	20 g.	5 g.
Other constituents:			
Difco Bacto-Agar	15 g.	15 g.	15 g.
Glass-distilled water to make one liter	+	+	+
Final pH	6.8	6.7	6.8

inoculation was made of the seed and the whole roots, excised on the fourth day from seed germinated on water agar, were grown on 100 ml. of the "nitrate-free" medium described in table 1, which was modified to contain only 0.5 per cent sucrose. An increase in the number of nodulated roots to 17 per cent was obtained in tips ranging in initial length from 25–45 mm. A nodule was even formed on a root grown from a 5-mm. tip initially excised on the third day (table 2).

(b) *Experiments with the new method of root culture.*—After these attempts it was concluded that probably no further improvement of nodulation could be achieved with the usual Petri dish method of root culture. The presence of sucrose in the medium surrounding the root encourages the proliferation of bacteria to such an extent that it was thought of little value to search for new chemical factors to increase nodulation until something could be done about the more basic problem of providing for the welfare of the root.

An answer was found in the use of the method of root culture developed by Raggio and Raggio (1956b) in which the root is supplied via its base with the organic moiety of the medium contained in solidified agar in a vial, while the rest of the organ lies in an inorganic, nitrate-free medium in a Petri dish in which inoculation with rhizobia is effected (fig. 1). This arrangement approaches the natural conditions of a root growing in the soil attached to the shoot; furthermore, the bacteria proliferate only to a limited extent in the immediate vicinity of the root, using as carbon and energy sources the sloughed-off dead cells (and perhaps

TABLE 2. *Experiments with isolated black wax bean roots grown on agar medium by the usual Petri dish method. The experiment of Lewis and McCoy (1933) is included for reference. S.E. = standard errors.*

Exp. No.	Mean no. of roots per culture	Total no. of roots	Mean final length & S. E. (mm.)	Mean final no. of laterals & S.E.	No. of nodules: On main axis	No. of nodules: On laterals	No. of roots nodulated	Mean no. of nodules/ nodulated root	Per cent of roots nodulated
Lewis & McCoy	3.0	60	?	?	4?		1	4.0	1.6
7	4.8	192	70 ± 5	?	0	9	5	1.8	2.6
22	3.3	143	65 ± 4	15 ± 2	8	7	12	1.2	8.4
20	2.4	48	55 ± 5	13 ± 2	1	8	8	1.1	16.6
20 (5-mm. tips)	4.5	50	30 ± 2	3 ± 0.5	0	1	1	1.0	2.0

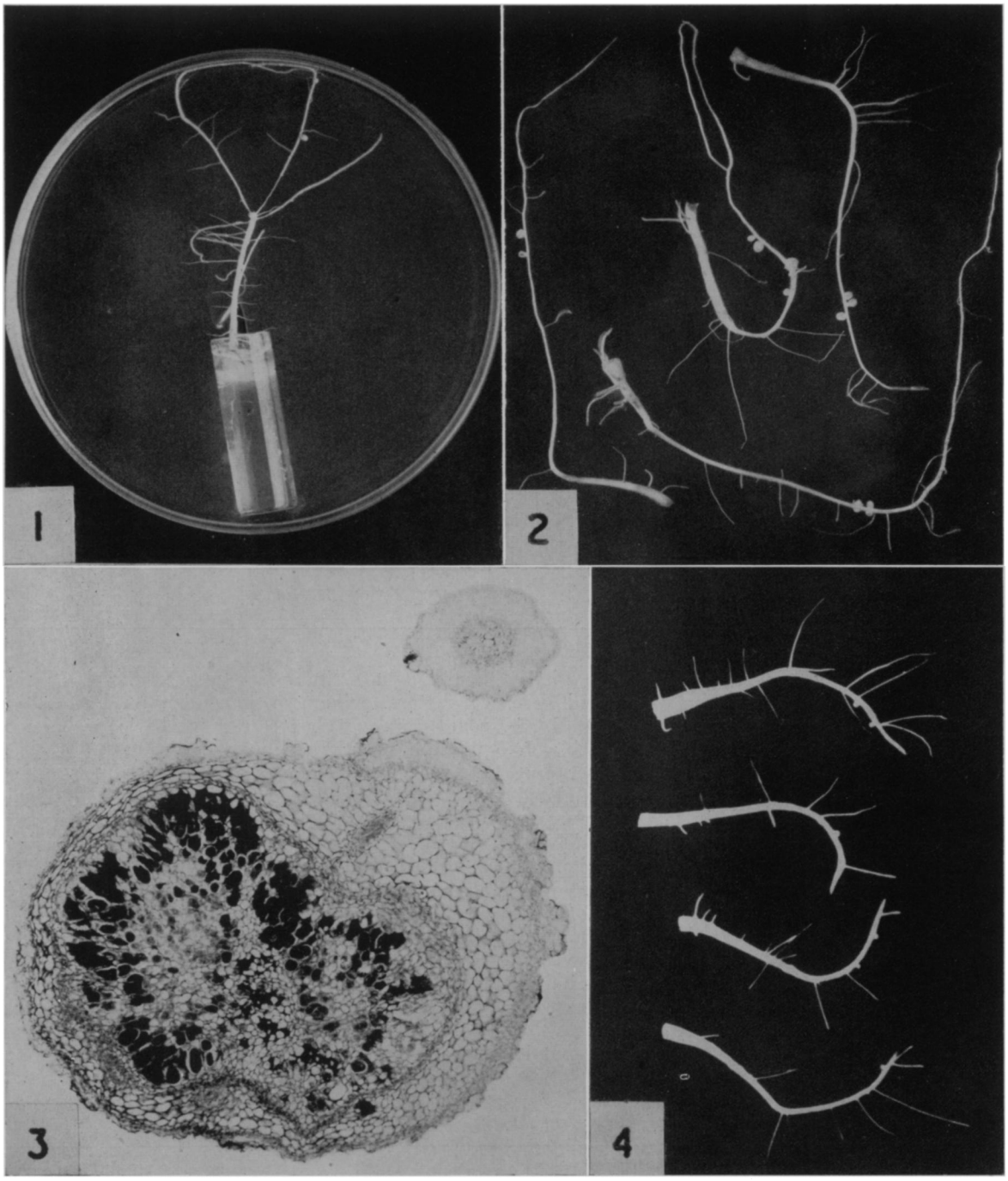

Fig. 1–4.—Fig. 1. Twenty-five day old inoculated black wax bean root (Exp. No. 32, table 3A, 2 per cent sucrose plus vitamins and glycine in the vial) showing 2 nodules.—Fig. 2. Some of the nodulated roots obtained in sand culture in Exp. No. 51 (10 per cent sucrose supplied in the vials). Roots have been removed from dishes and washed for photographing.—Fig. 3. Cross-section of an excised black wax bean root (upper right) showing a nodule in longitudinal section. Note large size of nodule relative to root diameter. Vascular strand in nodule tissue adjacent to the root is cut obliquely. ×58.—Fig. 4. Twenty-five-day-old excised inoculated soybean roots (Exp. No. 54; 2 per cent sucrose in vials) showing nodules.

excretions) of the root. Use of this procedure also circumvents the inhibition of nodulation by nitrate in the external medium, since the ion can be supplied, if required, via the root base. The following comparison illustrates the value of the method. Roots grown by the usual Petri dish method in a complete agar medium inoculated with bacteria 2 days after excision suffer a marked inhibition of growth (70 per cent in length and 90 per cent in number of laterals). Roots grown by the new method show no appreciable reduction in either length or number of branches upon inoculation in comparable conditions.

In the first experiment carried out with the new method (Exp. No. 32, table 3A), the inorganic constituents of the "nitrate-free" medium (table 1) were provided in a 1.5 per cent agar solidified medium in Petri dishes at pH 7.5. The organic constituents of the medium in the vials were varied in several ways with distilled water used as the control. In this experiment, whole roots 15–40 mm. long were used from seeds germinated on water agar for 4 days. The two following media supplied via the base each gave about 20 per cent nodulation: (1) the organic moiety of the "plus nitrate" medium in table 1 (fig. 1), and (2) five times the same organic moiety plus 1435.5 mg./l. $Ca(NO_3)_2 \cdot 4H_2O$ and 400 mg./l. KNO_3. Fifteen other treatments failed either to increase nodulation or to produce nodulation at all. The treatments included, in addition to those shown in table 3A: adding 0.5 or 5.0 g./l. powdered Difco yeast extract or adding nitrate in the vial; reducing sucrose to 0.5 per cent; shortening the initial tips to 10 mm.; varying the time of inoculation from immediately after to 4 days after excision; or decapitating the root tips. In all subsequent experiments to be described the following standardized procedure was adopted for securing excised roots. Seeds were germinated aseptically in the dark for 4 days on 1.5 per cent water agar in Petri dishes. Root tips 20 mm. in length were excised with a scalpel from roots which were between 25–40 mm. long. These roots were transferred with forceps to the culture dishes where they were inoculated with the rhizobial suspension 2 days after excision.

Because of the unpredictability of the results of these first experiments with the new method, other factors which might be limiting nodulation were investigated. Microscopic examination of cultured roots had repeatedly shown that roots grown on agar had only sparse and short root hairs. Thornton (1929) demonstrated that infection occurs preferentially through long root hairs and that the number of infected hairs relative to the total number of hairs is very small. Further, only a few of the infections ever develop into nodules. Therefore, any root with sparse and short root hairs is unlikely, on a simple statistical basis, to produce large numbers of nodules.

Other substrates with better physical properties for the inorganic medium were sought which might provide more favorable conditions for root hair development. One of the materials tried was vermiculite, which was spread in a thin layer in the Petri dish and was watered with 10 ml. solution of the inorganic constituents of the "nitrate-free" medium. The organic part was supplied in agar in the vials. Table 3B gives the results of Exp. No. 39. Nodulation of 44 per cent of the roots was obtained when only sucrose was provided in the vial and 74 per cent when, in addition to sucrose, vitamins and glycine were given via the base. The controls provided with water agar in the vial did not nodulate.

The striking improvement in per cent nodulation can be best explained on the basis of the tremendous increase in length and number of root hairs which attended the use of vermiculite. However, the presence in vermiculite of some chemical factor promoting nodulation could not be altogether discarded. To rule out the possibility of impurities, acid-washed sand was next tried as the substrate for the growth of roots in Petri dishes. Although it is not quite as good as vermiculite from the standpoint of its physical properties, washed sand offers the advantage of purity since, after thorough washing, it can be considered pure silica. Fifty g. of sand were added per dish and watered with 10 ml. of the "nitrate-free" inorganic medium. Excised roots were then provided organic constituents via the base as before. Representative results are presented in table 3C.

Under these conditions, some nodulation was always obtained when 2 per cent sucrose was supplied in the vials (values of two experiments: 6 and 7 per cent, e.g., Exp. No. 53), or when, in addition, vitamins and glycine were given (values of 4 experiments: 53, 9, 9 and 12 per cent, e.g., Exp. No. 51 and 53). No nodulation was obtained when water alone was supplied in one per cent agar (e.g., Exp. No. 53).

When 10 per cent sucrose was used in the vials together with vitamins and glycine, nodulation was increased to 71 per cent (Exp. No. 51, table 3C and fig. 2), a result comparable to that obtained with vermiculite. Addition of nitrate via the base yielded a percentage of nodulation (58 per cent) which did not differ significantly from that obtained with sucrose, vitamins and glycine alone. However, when nitrate was given in the inorganic medium in the dish (Exp. No. 51), a significant inhibition of nodulation (only 31 per cent) occurred. A photomicrograph of a section of a nodule obtained in Exp. 51 is shown in fig. 3.

In the experiments which followed, 10 per cent sucrose was routinely used in the vials and the levels of glycine and vitamins were likewise increased five-fold over those in the medium in table 1. Also, in view of the fact that high levels of calcium and phosphorus and a pH close to neutrality have been repeatedly shown to be beneficial

TABLE 3. *Experiments with isolated black wax bean roots grown in agar, vermiculite or sand using the new method of root culture. Substances in the vials were in concentrations 1, 5, or 10 times (+, 5×, 10×) those of the "Plus nitrate" medium of table 1. Inoculation of roots was with Rhizobium phaseoli, strain 17. Dash = zero. S.E. = standard errors.*

Exp. No.	Medium in dish	Substances in vial: Sucrose %	Vitamins + Glycine	Yeast 0.5 g./l.	Nitrate	No. of roots used	Mean final length & S. E. (mm.)	Mean final no. of laterals & S.E.	No. of nodules: On main axis	No. of nodules: On laterals	No. of roots nodulated	Mean No. of nodules per nodulated root	Per cent of roots nodulated
						A. AGAR							
32	"Nitrate-free"	—	—	—	—	17	75 ± 1.9	4 ± 0.9	—	—	—	—	—
	(inorganic)	2	+	—	—	17	125 ± 10.1	22 ± 3.0	1	4	3	1.7	18
		10	5×	—	—	17	110 ± 5.2	26 ± 2.3	—	—	—	—	—
		20	10×	—	—	14	100 ± 1.6	24 ± 2.2	—	—	—	—	—
		10	5×	—	5×	14	127 ± 8.5	41 ± 3.1	1	2	3	1.0	21
						B. VERMICULITE							
39	"Nitrate-free"	2	—	—	—	18	93 ± 8.9	13 ± 1.9	7	13	8	2.5	44
	(inorganic)	2	+	—	—	19	130 ± 6.9	21 ± 2.2	14	26	14	2.8	74
						C. SAND							
51	"Nitrate-free"	2	+	+	—	11	64 ± 5.7	10 ± 1.8	1	—	1	1.0	9
	(inorganic)	10	+	—	—	14	83 ± 4.8	10 ± 0.7	20	2	10	2.2	71
		10	+	—	+	12	84 ± 7.2	13 ± 1.8	6	8	7	2.0	58
51	"Plus nitrate"	2	+	+	—	12	70 ± 5.2	17 ± 1.8	1	—	1	1.0	8
	(inorganic)	10	+	—	—	13	68 ± 5.0	13 ± 1.4	4	6	4	3.3	31
53	"Nitrate-free"	—	—	—	—	16	32 ± 3.0	—	—	—	—	—	—
	(inorganic)	2	—	—	—	14	82 ± 8.0	14 ± 1.7	3	—	1	3.0	7
		2	+	+	—	16	64 ± 2.9	8 ± 0.7	1	1	2	1.0	12
60	"O"	10	—	—	—	20	71 ± 2.8	10 ± 0.8	4	9	8	1.6	40
	(inorganic)	10	+	—	—	22	64 ± 4.3	9 ± 0.5	2	23	10	2.5	45
		10	+	—	—	20	84 ± 6.9	14 ± 1.1	2	45	17	2.7	85
	"O" (complete)	No vial				20	46 ± 1.4	4 ± 0.8	—	1	1	1.0	5
61	"O"	10	—	—	—	22	71 ± 5.5	12 ± 1.1	4	35	11	3.5	50
	(inorganic)	10	+	—	—	22	75 ± 5.0	11 ± 0.7	11	21	16	2.0	72
		10	+	—	+	20	100 ± 9.0	14 ± 1.2	7	38	14	3.2	70
		10	—	+	—	22	74 ± 4.8	12± 1.1	6	16	11	2.0	50
	"O" (complete)	No vial				14	50 ± 4.0	7 ± 0.9	—	2	1	2.0	7

to both nodulation and nitrogen fixation (Wilson, 1940), the liquid medium in sand in the dish was changed from the inorganic part of the "nitrate-free" medium to the inorganic part of medium "O" (table 1). In addition, certain experiments were carried out with roots grown in sand to which had been supplied the complete "O" medium, including the organic components. These tests represent, in fact, a variation of the usual Petri dish root culture method. The results obtained are shown in table 3C (Exp. No. 60 and 61).

In the first place, it is evident that excised roots grown in sand but surrounded by the complete culture medium "O," including the organic components, grow poorly and show very little nodule formation, even despite the excellent physical substratum provided by the sand. In contrast, all experiments carried out with the new method, in which the sucrose and nitrate are provided from the vial, show high percentages of nodulation. In experiments in which roots were fed 10 per cent sucrose alone, 40 to 50 per cent nodulation was obtained; the further provision of vitamins and glycine in addition to 10 per cent sucrose further increased nodulation to an average of about 70 per cent. Provision of nitrate via the base did not significantly increase this percentage. Nodules formed on roots supplied with nitrate via the base were in fact of smaller size. Yeast extract added to sucrose in the vial appeared to have no beneficial effect.

Throughout all of the experiments reported here, measurements of the pH of the media, both in the vial and in the dish, were made at the beginning and end of the experiment. Good nodulation occurred over a wide range of pH as measured by the final pH of the medium in the dish, which varied from 3.8 to 7.5. No correlation was evident between pH of the medium and percentage of roots nodulated. In Exp. No. 60 and 61, table 3C, where consistently high percentages of nodulation are reported, the pH of the medium in the dish was maintained at 7.5.

One further fact should be noted. The percentage of roots which form nodules in a given experiment is one measure of proper conditions for nodulation. In the various changes in experimental conditions already described, the percentage of roots nodulated had consistently increased. A second measure of good conditions for nodulation is that of the mean number of nodules per root. This figure had also consistently increased as conditions were improved, reaching a figure as high as 3.5 nodules per root in certain experiments.

On a dry-weight basis, nodulation of isolated roots in culture is of the same order as that of seedling roots. Thus, in a typical experiment, the mean number of nodules per isolated root was 2.5, the mean dry weight per root was 7 mg., so that the mean number of nodules per 10 mg. dry weight of root was 3.6. The comparable figures for light-grown shootless seedlings (Raggio and Raggio, 1956a) were: a mean number of 5.6 nodules per root with mean dry weight of 39 mg. per root. The mean number of nodules per 10 mg. dry weight in seedling roots was thus 1.4, a figure actually slightly below that of isolated roots.

The nodulation of isolated roots of soybean.—In view of the excellent results obtained with the new method of excised root culture in nodulation of isolated black wax bean roots, it was thought desirable to test another leguminous plant species belonging to a different cross-inoculation group. Since soybean seedlings require only the cotyledons for nodulation (Raggio and Raggio, 1956a), it was predicted that, as in the case of black wax bean, nodulation should be obtained on isolated roots if the inhibitions by nitrate and sucrose were obviated.

Experiments were carried out using 20-mm. root tips cut from seeds that had germinated 4 days on water agar. They were cultured using the sand technique and were inoculated 2 days after excision with strain 59 of *Rhizobium japonicum*. Measurements of roots and counts of nodules were made after 25 days (table 4).

Although the growth of the roots in the "nitrate-free" medium supplied with 2 per cent sucrose via the base was poor (the tips showed necrosis), 47 per cent of the roots were nodulated (fig. 4). With 10 per cent sucrose provided via the base and the inorganic part of medium "O" surrounding the roots, growth was better, but no increase in nodulation occurred. When vitamins plus glycine, nitrate, or yeast extract were supplied via the base, no effect on nodule formation was evident. Roots which were grown by a variation of the usual Petri dish method with the complete medium "O" (organic plus inorganic moieties) provided in the sand showed no nodulation. Although the growth in length of these roots was not affected, their branching was strongly inhibited (Exp. No. 62, table 4).

DISCUSSION.—With isolated roots grown by the usual Petri dish method and inoculated immediately after excision (table 2) it has been confirmed that nodulation, even if only about 5 per cent, is possible. A higher per cent nodulation (17 per cent) was obtained when inoculation was made of seed (Exp. No. 20) prior to culturing the excised root by the usual method. This result might be expected since the bacteria probably had a chance to infect the root during the period of 4 days before excision and transfer to the growing medium. This is corroborated by the fact that in Exp. No. 20 there occurred the single case in all experiments of nodulation of a root derived from a 5-mm. tip.

The separation of the organic and inorganic moieties of the medium made possible by the new method of root culture was a definite improvement, since with two different media in the vials (table

TABLE 4. *Experiments with isolated soybean roots grown by the new method in sand. Substances in the vials were in the concentrations of the "Plus nitrate" medium of table 1, except nitrate which was 5 times as concentrated. Dash = zero. S.E. = standard errors.*

Exp. No.	Medium in dish	Substances in vial: Sucrose %	Vitamins + Glycine	Yeast 0.5 g./l.	Nitrate	No. of roots used	Mean final length & S.E. (mm.)	Mean final no. of laterals & S.E.	No. of nodules: On main axis	No. of nodules: On laterals	No. of roots nodulated	Mean No. of nodules per nodulated root	Per cent of roots nodulated
54	"Nitrate-free" (inorganic)	2	—	—	—	15	43 ± 1.8	7 ± 0.5	9	—	7	1.3	47
		2	+	—	—	15	43 ± 2.0	8 ± 0.4	8	—	6	1.1	40
		2	+	+	—	15	43 ± 1.9	9 ± 0.7	7	—	7	1.0	47
62	"O" (inorganic)	10	—	—	—	19	55 ± 4.0	15 ± 1.2	9	3	9	1.3	47
		10	+	—	—	20	65 ± 2.1	16 ± 0.7	16	1	10	1.7	50
		10	—	+	—	22	60 ± 2.3	14 ± 1.1	15	—	10	1.5	45
		10	+	—	+	18	62 ± 2.0	14 ± 1.1	9	1	7	1.4	39
62	"O" (complete)	No vial				22	59 ± 4.1	1 ± 0.4	—	—	—	—	—

3A) consistent nodulation of about 20 per cent was obtained on excised whole roots inoculated 3 days after excision. The increase in nodulation thus gained is to be attributed to the physical separation of the medium into its organic and inorganic parts. This separation circumvents both the excessive growth of bacteria and the inhibition of nodulation by sucrose. When the solidified agar in the dish containing the inorganic medium was changed for a substrate of vermiculite or sand, a sharp rise in nodulation ensued (table 3B, C), an effect attributed to better root hair development.

With respect to root hair formation on inoculated excised roots grown on agar by the conventional method, Lewis and McCoy (1933) reported many long hairs on black wax bean roots. In the present work consistently poor root hair formation was obtained with 5, 10 or 20-mm. root tips of black wax bean grown on agar by any of the methods. McGonagle (1944) also found very sparse and short root hairs on inoculated cultured pea roots. This interesting aspect of the problem presents many unanswered questions which point to the desirability of investigating the factors involved in root hair formation, in general.

From the low nodulation obtained on excised roots grown in the complete medium in sand culture (table 3C), it can be concluded that the physical conditions provided by the sand are not enough by themselves for nodulation and that their beneficial effect is exerted only when, by the use of the new method, the bacteria and most of the root surfaces are prevented from coming in direct contact with sucrose.

In the light of the results of the present investigation, the very low nodulation obtained by Lewis and McCoy (1933) and the negative results of Seppilli et al. (1941) and McGonagle (1944) can be ascribed to the summation of three negative factors: (1) poor conditions for root growth due to exaggerated bacterial multiplication in the presence of sucrose; (2) inhibition of nodulation by sucrose, and (3) a poor physical substrate for root hair production.

In both of the plant species studied here it has been demonstrated (Raggio and Raggio, 1956a) that materials contained in the cotyledons of seedlings are required for nodulation of the roots. One might conclude from the present experiments with isolated roots that a proportion of the 20-mm. tips have received from the cotyledons the required materials for nodule formation, since they will nodulate when supplied with sucrose alone via their bases. The fact that the addition of vitamins and glycine increased appreciably the percentage of nodulation of such roots gives evidence that a percentage of tips is deficient in these factors. However, no differentiation between growth factors and nodulation factors is apparent in the experiments taken as a whole, since adding vitamins plus glycine increases nodulation but not growth,

while supplying nitrate via the base increases growth but not nodulation. An important experiment for the future, and a better way to study the biochemical factors concerned in nodulation, will be an investigation of nodule formation in isolated roots depleted through successive transfers. Preliminary experiments indicate that black wax bean roots will not grow satisfactorily through successive transfers in the modified White's medium by the methods used here. This fact means that the nutritional conditions required by the root for normal growth are not provided by the medium used. A more detailed study of the nutrition of this species or, alternatively, the use of other species which are already known to grow in subtransfers, would be of interest.

As shown in Exp. No. 51 (table 3C), nitrate when supplied to the medium in the dish inhibits nodulation of isolated roots grown by the new method. Supplying nitrate via the root base, on the other hand, has no such inhibitory effect. The lack of inhibition of nodulation by nitrate supplied via the base is to be expected, however, since the effect of nitrate was shown to be localized to the place of application by Wilson (1917). Thornton (1936) reported that nitrate inhibits the action of bacterial secretions claimed to increase the length, number, curling and infection of root hairs.

One striking difference between the nodulation of seedlings and of isolated roots of black wax bean is that much greater numbers of nodules are formed on the primary root axis in isolated roots compared to seedling roots. For example, out of 200 nodules counted on seedling roots (Raggio and Raggio, 1956a), only 7 occurred on the primary roots. All others occurred on lateral roots. On the other hand, of the 446 nodules obtained in all the experiments with isolated roots, 104 occurred on primary roots. At the present stage of our knowledge, no explanation can be given, since in the various treatments reported here, no consistency in behaviour with respect to the media provided is evident.

In the experiments with isolated soybean roots (table 4), as in the case of black wax bean, the failure to obtain nodulation in sand culture with the usual Petri dish method points out once more that the beneficial effect of a physical substrate favorable to root hair formation is only elicited in a system where the organic and inorganic moieties of the nutrient medium are maintained physically separated.

SUMMARY

Nodulation of isolated black wax bean roots grown in vitro on an agar nutrient medium by the usual method of root culture in Petri dishes was found to be very poor, with only about 5 per cent of the roots inoculated with the effective bacterial strain producing nodules. By a progressive series of changes in the techniques of root culture a consistently high percentage of nodulation of isolated roots was achieved. Use was made of a recently developed method in which the root is supplied with the organic materials of the medium via its base while bacterial inoculation is made of the root growing in a nitrate-free inorganic medium. With agar as the substrate for the inorganic medium, poor root hair development occurred but nodulation increased to 20 per cent. When agar was replaced by vermiculite or sand as the substratum for the inorganic medium, root hair formation was improved and nodulation increased to 75 per cent. Twenty-mm. root tips of black wax bean, excised from seeds germinated for 4 days in water agar, when cultured in sand in an inorganic medium lacking nitrate, but provided sucrose, vitamins and glycine via their bases, consistently showed a high percentage of nodulation. Sucrose alone was less effective in causing nodulation. Nitrate added to the inorganic medium significantly reduced nodule formation; nitrate supplied via the base exerted no such inhibitory effect. The number of nodules per unit dry weight of root was of the same order as that obtained with seedlings. However, many more nodules are formed on the main axis of isolated black wax bean roots than in seedling roots with the strain of *Rhizobium* used. When sucrose alone was supplied to excised soybean roots via the root bases in conditions comparable to those described for black wax bean roots, about 50 per cent nodulation was obtained. The addition of vitamins plus glycine or yeast extract did not further increase nodulation. Isolated roots of both species failed to nodulate when only distilled water in agar was supplied via the bases. Thus, with a technique which (a) prevents sucrose from encouraging bacterial growth to the detriment of the root; (b) circumvents the inhibition of nodulation by nitrate and sucrose, and (c) provides a good substrate for adequate root hair formation, the nodulation of isolated leguminous roots can be readily achieved.

DEPARTMENT OF BOTANY,
UNIVERSITY OF CALIFORNIA,
BERKELEY 4, CALIFORNIA

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GROWTH AND CONTROLLED MORPHOGENESIS IN PEA ROOT CALLUS TISSUE GROWN IN LIQUID MEDIA[1]

John G. Torrey and Yoshio Shigemura

IN RECENT YEARS considerable interest has centered in the nutritional requirements of isolated fragments of higher plant tissues cultured in vitro. From the early work of White (1943) and of Gautheret (1942), it has been known that tissues derived from the immediate morphological region of the vascular cambium of either stem or root are particularly susceptible to culture in a suitable nutrient medium. Much information has been gained concerning the specific nutritional requirements of a number of callus tissues derived from the vascular cambium region of a wide variety of plants (Gautheret, 1955b).

Most of the callus tissues which have been cultivated in vitro have been derived from secondary tissues of stems. Relatively few callus tissues of root origin have been studied. One notable exception is carrot tissue which has been grown extensively in culture, but even here precise distinction between root and stem (hypocotyl) tissue is difficult to make and frequently has not been made. Only in a few cases have root callus tissue cultures of distinctly root origin been described. Skoog (1944) reported the spontaneous formation of root callus by cultured roots from hybrid tobacco tissues in culture. Jagendorf et al. (1952) describe a callus tissue produced from cabbage roots, Kandler (1950) from sunflower roots in culture, Tryon (1955) from tobacco roots, Nickell (1955) from roots of sweet clover, and Black (1947) and Nickell (1954) the abnormal virus tumor tissue produced by sorrel roots.

In studying problems of plant morphogenesis using tissue culture methods, careful identification of both morphological and anatomical origin of the tissue may be a matter of considerable importance. Thus, callus tissue derived from roots may be expected to differ fundamentally in inherent morphogenetic capacities from stem callus tissue. These differences may be perpetuated in culture or they may be modified by manipulation of the medium or by the passage of time.

During the past several years, studies have been made of the particular nutritional conditions necessary for continued meristematic activity in the root meristem of isolated pea roots grown in vitro (Torrey, 1954) and for the initiation of cell divisions leading to lateral root formation in isolated pea root segments (Torrey, 1956). Because of our interest in cell division and its biochemical control in pea roots grown in culture, it appeared desirable to us to isolate tissues from the vascular cambium region of pea roots and to study the nutritional conditions necessary for continued cellular divisions in such tissues. Once established in culture as a root callus tissue, it was hoped that the peculiar conditions giving rise to the initiation and continued activity of the secondary meristem in vivo might be discovered and that a study of the inherent morphogenetic capacities of the root callus tissue itself might be made. In the experimental work to be reported here, an account is given of the establishment on a complex nutrient medium of a callus culture from tissues isolated from the vascular cambium region of pea roots. During the course of this study, the callus tissues have been grown in both solidified agar media and in liquid media with constant agitation. Under the latter conditions two morphologically distinct tissues have become evident whose occurrence is dependent upon the constituents of the nutrient medium.

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