NODULATION AND GROWTH OF *TRIFOLIUM SUBTERRANEUM* L. cv. MOUNT BARKER IN AGAR CULTURE

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Summary

The presumptive effect of root exudates on the course of nodulation of *Trifolium subterraneum* L. cv. Mount Barker in agar culture was investigated by means of the preplanting technique. The technique was also re-examined with a view to its use for the bioassay of substances influencing nodule formation. Preplanting was found to remove unidentified nitrogenous compounds from the agar media. It was also found that some of the data obtained are subject to observer effects.

The environmental conditions and the type of agar used to prepare the root media have large and significant effects on the number of nodules formed, but the effect of preplanting is slight and varies in direction with the type of agar. There is no acceptable evidence that any of these factors affects the interval between inoculation and initiation of nodulation.

Plant yield was affected in a highly significant manner by the environmental conditions but responses to the type of agar and preplanting were confined to plants grown in the glasshouse.

It was concluded that further physiological investigations are required before chemical fractionation of the root exudate of this species is warranted.

I. Introduction

To study the effects of root exudates on the nodulation of legumes, Nutman (1953, 1957) developed a preplanting technique whereby a donor plant was grown on a nutrient agar slope for a period during which organic substances were exuded from the roots and accumulated in the agar medium. The donor plant was removed at the end of this preplanting period and a legume, inoculated with a *Rhizobium* species, was sown in its place. The course of nodulation of the test species was then observed and compared with that of a plant growing on the untreated medium.

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Nutman found that, in general, preplanting accelerated the appearance of the first nodule, but that over longer periods, fewer nodules were formed. However, these effects were sometimes inconsistent and varied with the species, or genetic line, of plant and bacterium.

Later, Gibson and Nutman (1960) re-examined these effects and found that the early stimulation of nodulation of several legumes was due to the removal, by the donor plant, of nitrates derived from the tap water used to prepare the agar media. It was also found that the removal of nitrates largely accounted for the later inhibition of nodulation produced by preplanting such media. However, in other experiments these authors found that preplanting in a presumed nitrogen-free medium hastened initial nodulation to a slight extent and lowered the number of nodules on the test plants by 20–40%. They attributed the latter effect to an inhibitor present in the root exudate, but considered that the slight stimulation of nodulation was more likely to be due to the type of agar, the inorganic composition of the medium, or the time of inoculation, than to root exudates.

Kefford, Brockwell, and Zwar (1960) detected tryptophan in the root exudate of *Trifolium subterraneum* L. cv. Mount Barker growing in water culture, and obtained evidence that this substance is converted into indolyl-3-acetic acid by several *Rhizobium* species. From a study of the course of nodulation of varieties of *Medicago sativa* L. and *M. tribuloides* Desr. growing in agar media to which tryptophan had been added, these authors concluded that inhibitory preplanting effects arise from accumulation of auxin in the system. However, on the basis of other experiments with *Phaseolus vulgaris* L. and *Pisum sativum* L., Radley (1961) suggested that the inhibitory substance exuded from nodules and root tips may be a gibberellin. On the other hand, Valera and Alexander (1965) found that the root exudate of *M. sativa* did not affect the nodulation of excised roots of that species.

The present experiment was designed to define more precisely the effects of root exudates on the course of nodulation and growth of *T. subterraneum* cv. Mount Barker growing in an agar culture inoculated with an effective strain (SU297) of *Rhizobium trifolii* Dang. The work was restricted to this legume, which was chosen primarily because it nodulates promptly and sparsely under these conditions.

It was considered necessary to study the influence of environment on these effects because Rovira (1959) found that both the composition and quantity of root exudate obtained from another cultivar (Bacchus Marsh) of this species varies with the temperature and the light intensity. Because the unidentified nitrogenous compounds present in agar might influence the course of nodulation (Gibson and Nutman 1960), it was also considered necessary to study the effects of using two root media prepared from samples of agar which differed widely in nitrogen content.

As it was hoped that the preplanting technique might serve as the basis of a bioassay procedure to be used in subsequent chemical fractionation of the root exudate, this was standardized by selecting uniform plant material by statistically acceptable methods and by refining some aspects of the experimental technique. The experimental data were collected independently by two persons, so that any observer effects could be recognized.

II. Experimental

(*a*) *Growing Conditions*

The experiment was carried out during the period July 6–20, 1959 (preplanting period), and July 27 to September 14, 1959 (test period).

The unit providing the controlled environment consisted of a chamber, constructed of plywood on a timber frame, which was divided horizontally by a sheet of plate glass. The ventilated upper compartment was lined with aluminium foil and contained 10 fluorescent lamps (Atlas Warm White, 4 ft, 40 W). The lower compartment had insulated walls and all interior wooden surfaces were coated with a high-gloss white paint. By means of an air-conditioning unit (Kelvinator H625, 2000 W) and suitable sheet metal ducts, air was circulated continuously through the lower compartment. The air temperature was controlled at 70 ± 2°F during a 16-hr photoperiod and at 60 ± 2°F during an 8-hr nyctoperiod.

Plants were grown in Pyrex test tubes (6 by $\frac{3}{4}$ in.), accommodated in solid wooden racks containing two rows of 12 equidistant holes (1 in. diam., 2 in. deep), the holes in each row being staggered with respect to the holes in the other row. The racks were placed side by side on sliding platforms which could be withdrawn from the lower compartment.

The lamps were allowed to age for 10 days before the plants were placed in the unit; the light intensity at the level of the racks was then found to be *c.* 1000 f.c., except towards the ends of the compartment where a slight drop in intensity was noted. In order to distribute this difference (*c.* 20 f.c.) over the whole treatment, racks were moved three positions in a clockwise direction daily.

In the glasshouse, plants were illuminated by natural light only. During the preplanting and test periods, day lengths averaged *c.* 10 and 11 hr, respectively. During the combined photoperiods, the mean temperature was 85°F, and during the nyctoperiods, 51°F.

(*b*) *Preparation of Media*

Judex shredded agar [found (Kjeldahl): N, 0·29%] and Difco Noble granular agar [found (Kjeldahl): N, 0·07%] were selected for the preparation of the two media. The appropriate agar (12 g), K_2HPO_4 (0·5 g), KH_2PO_4 (0·5 g), precipitated $Ca_3(PO_4)_2$ (0·4 g), $FePO_4$ (0·13 g), NaCl (0·1 g), $MgSO_4.7H_2O$ (0·2 g), $FeCl_3$ (0·01 g), and the trace element solution (1 ml) described by Gibson and Nutman (1960), were taken up in glass-distilled water (1 litre). The seedling nutrient solution consisted of the same quantities of these salts in 4 litres of glass-distilled water.

After dispensing the agar media (8 ml per test tube) the tubes were closed with rolled cotton-wool plugs, autoclaved at 15 lb/in^2 for 20 min, then sloped so that the agar extended $3\frac{1}{2}$ in. above the bottom of the tube. 48 tubes (comprising 6 control slopes of each agar and 18 slopes of each agar to be preplanted) were then arranged in random order in a wire basket (6 by 6 by 6 in.), with one blank tube inserted in the remaining space.

(c) *Selection and Surface-sterilization of Seeds*

Individual seeds (*c.* 1400) weighing between 5·8 and 6·4 mg were selected from a commercial sample of *T. subterraneum* cv. Mount Barker seed (1959 Store, No. 1095, obtained from F. H. Brunning Pty. Ltd., Melbourne) of mean weight 6·1 mg. To avoid damage, seeds were handled with forceps, the tips of which were sheathed with narrow rubber tubing. The seeds were sterilized in batches of *c.* 350 by immersion in ethanol (50 ml) for 15 sec followed by 4½ min in an aqueous solution of mercuric chloride (0·1%, 50 ml). The seeds were then washed quickly with three changes of sterile glass-distilled water, allowed to stand in water for 5 min, washed again in the same manner, and then given eight single washings at intervals of 15 min. Excess water was drained from the swollen seeds, which were then stored at 40°F for 43 hr.

TABLE 1

DRY WEIGHT AND NITROGEN CONTENT OF SEEDS AND 16-DAY-OLD UNINOCULATED DONOR PLANTS

Plant Material	Type of Agar	Environment	Mean Weight per Plant (mg)	Mean Weight of Nitrogen per Plant (mg)	Increase in Total Nitrogen over Seed Reserve during Growth (%)
Seeds (unsterilized)			6·2	0·324	
Seeds (sterilized)			6·1	0·321	
Seedlings	Shredded	Controlled	10·0	0·380	18·4
Seedlings	Granular	Controlled	9·1	0·327	1·9
Seedlings	Shredded	Glasshouse	8·9	0·376	17·1
Seedlings	Granular	Glasshouse	8·7	0·332	3·4

(d) *Sowing and Selection of Donor Plants*

The 1080 tubes which were to contain unselected donor plants were each sown with one seed placed *c.* 1 in. from the top of the agar slope with the micropyle downwards. Care was taken to preserve the random order of the tubes in the wire baskets, and all manipulations in the tubes were carried out aseptically in the usual manner. The wire baskets were then enclosed in black plastic envelopes and placed at random in a dark cabinet at 67°F. After 44 hr *c.* 90% of the tubes contained vigorous seedlings; almost all of the remainder contained a seed which had fallen to the bottom of the tube.

The tubes were removed from the wire baskets in the order in which they had been sown and arranged in one of four wooden racks so that the tubes of each treatment formed a continuous sequence. Consecutive groups of three tubes were then ranked in order of increasing radicle length and the two outer tubes discarded, leaving a total of 360 tubes containing donor plants. These, and 360 unsown control tubes were then numbered at random within each treatment, reassembled in numerical order, then placed in wooden racks in a predetermined random arrangement. This step was designed to distribute at random within each treatment any handling effects which may have arisen during the above procedures, some of which occupied several hours.

(*e*) *Preplanting*

After addition of seedling nutrient solution (1·5 ml) to each tube, 10 racks (containing a total of 30 replicates of the proposed final treatments) were placed in the controlled environment and 20 racks placed in the glasshouse. (The capacity of the controlled environment limited the number of replicates to 30 per treatment.) Sixteen days after sowing, the donor plants were removed completely with minimum disturbance of the agar slopes. The tubes of each final treatment were then distributed equally between, and in random order within, wire baskets which were stored in the dark at room temperature. Each donor plant, including the seed coat, was wrapped in an envelope of filter paper (Whatman No. 542) and dried for 90 min in an oven set at 105°C. The donor plants of each treatment, untreated seeds, and surface-sterilized seeds were then assembled in groups of 10 and analysed for total nitrogen content by the microKjeldahl method. The results of these analyses are set out in Table 1.

(*f*) *Selection and Establishment of Test Plants*

Seeds were selected, surface-sterilized, refrigerated, and sown in both pre-planted and control tubes as described above. After germination as before, seedlings having radicles less than 4 mm long and tubes containing broken or contaminated agar slopes were discarded. The required number of replicate seedlings per treatment (20 or 40) were then selected at random from the remainder. The liquid in each tube was adjusted to the same level by addition of sterile glass-distilled water and then seedling nutrient solution (0·5 ml) containing *c.* 10^7 viable *Rhizobium trifolii* strain SU297 was added. The bacterial suspension was allowed to wash over each root system then the tubes were assigned to predetermined random positions in the racks. Ten racks were placed in each environment and remained there until 51 days after sowing.

At 24 and 39 days after sowing, the level of liquid in each tube was adjusted to the original level with sterile glass-distilled water.

(*g*) *Observations of Initial Nodulation*

Plants were first examined for the presence of nodules 6 days after sowing and thereafter, daily. Structures suspected to be nodules were noted and, if later confirmed, the time to initiation of nodulation was taken as the interval elapsing between sowing and the first observation. Throughout the experiment each plant was examined independently by two observers (I and II): the results are set out in Table 2.

(*h*) *Observations on Total Number of Nodules*

51 days after sowing, the contents of each tube were removed and the roots of each plant freed by breaking up the agar slope gently under water. The plants were then placed in Petri dishes containing water and the nodules present on every root system were counted independently by both observers. The results are recorded in Table 3. The dry weight of each plant (including the seed coat) was then determined as described above; the results are recorded in Table 4.

III. Results

(*a*) *Statistical Treatment and Presentation*

As there was no evidence that the environment during preplanting had any effect on the test plants, all tables have been reduced by grouping over this variable.

Table 2

MEAN TIME INTERVAL (IN DAYS) BETWEEN SOWING AND INITIATION OF NODULATION OF *T. SUBTERRANEUM* CV. MT. BARKER IN AGAR CULTURE

Observer	Type of Agar	Controlled Environment		Glasshouse		General Means	
		Preplanted	Control	Preplanted	Control	Preplanted	Control
I	Shredded	10·6	10·2	10·4	10·7	10·5	10·5
	Granular	11·0	10·6	10·8	10·3	10·9	10·4
II	Shredded	10·3	10·0	10·7	10·6	10·5	10·3
	Granular	10·7	10·3	10·7	10·5	10·7	10·4

Analysis of Variance

Source of Variation	Observer I		Observer II	
	Degrees of Freedom	Mean Square	Degrees of Freedom	Mean Square
Agar	1	4·03	1	3·20
Preplanting	1	7·01*	1	5·29
Agar × preplanting	1	4·26	1	0·21
Environment of donor plant	1	0·86	1	3·22
Environment of test plant	1	0·51	1	4·67
Environment of donor plant × environment of test plant	1	0·73	1	8·07*
Other interactions	9	3·20	9	2·03
Error	441	1·64	443	1·43

* $P < 0{\cdot}05$.

During the experiment each observer rejected certain test plants which were judged to be aberrant in some respect, and in some cases, a plant acceptable to one observer was rejected by the other. Statistical analysis of the dry weights of plants supported most of the observers' subjective decisions. Data for plants defined as statistically acceptable on this criterion have been identified as III in Tables 3 and 4. The degree of agreement in rejection of plants between I, II, and III is indicated in Table 5.

In Tables 2 and 3, data concerning the nodulation of plants acceptable to each observer are presented separately. In addition, the data on the total number of nodules on statistically acceptable plants (III) has been examined (Table 3) using the average estimate of I and II.

The omission of tubes from some treatments rendered the analysis non-orthogonal. Means of treatments were determined and totals computed for the full

TABLE 3

MEAN LOGARITHM OF NUMBER OF NODULES FORMED PER PLANT OF *T. SUBTERRANEUM* CV. MT. BARKER AFTER GROWTH FOR 51 DAYS IN AGAR CULTURE

Observer	Type of Agar	Controlled Environment		Glasshouse		General Means	
		Preplanted	Control	Preplanted	Control	Preplanted	Control
I	Shredded	0·724	0·663	1·162	1·171	0·943	0·917
	Granular	0·809	0·857	1·263	1·328	1·035	1·093
II	Shredded	0·707	0·640	1·134	1·123	0·921	0·882
	Granular	0·811	0·849	1·219	1·296	1·015	1·073
III (mean of I and II)*	Shredded	0·716	0·652	1·148	1·147	0·932	0·899
	Granular	0·810	0·852	1·241	1·312	1·025	1·082
Difference of I and II*	Shredded	0·018	0·023	0·029	0·048	0·023	0·035
	Granular	1·998	0·009	0·044	0·032	0·021	0·020

Analysis of Variance

Source of Variation	Mean Square			Observer Contrast	
	I	II	III	Source	Mean Square
				Observer	0·1486***
Agar	2·3340***	2·3590***	4·5761***	Observer × agar	0·0046
Preplanting	0·0562	0·0112	0·0359	Observer × preplanting	0·0020
Agar × preplanting	0·1574**	0·2962***	0·4793***	Observer × agar × preplanting	0·0023
Environment	26·9417***	23·3712***	49·5950***	Observer × environment	0·0413***
Agar × environment	0·0141	0·0168	0·0201	Observer × agar × environment	0·0040
Preplanting × environment	0·0282	0·0661	0·1262	Observer × preplanting × environment	0·0003
Agar × preplanting × environment	0·0073	0·0043	0·0174	Observer × agar × preplanting × environment	0·0052
Error	0·0171	0·0188	0·0349	Error	0·0016

* For plants defined as statistically acceptable. **$P<0·01$. ***$P<0·001$.

complement of tubes for the treatment (i.e. 20 or 40) and the analysis was then made on these totals and nominal tube numbers in the conventional manner. Because the main effects and interactions were thereby inflated relative to the replicate variance, an adjustment was made to the error term by increasing it by the expectation of the variance between treatments based on the augmented numbers relative to the actual numbers. This increase was of the order of 5%.

TABLE 4

DRY WEIGHT (LOGARITHM OF MILLIGRAMS) PER NODULATED PLANT OF *T. SUBTERRANEUM* CV. MT. BARKER AFTER GROWTH FOR 51 DAYS IN AGAR CULTURE

Observer	Type of Agar	Controlled Environment		Glasshouse		General Means	
		Preplanted	Control	Preplanted	Control	Preplanted	Control
I	Shredded	1·534	1·535	1·341	1·365	1·438	1·450
	Granular	1·530	1·531	1·342	1·350	1·436	1·440
II	Shredded	1·530	1·535	1·341	1·365	1·436	1·450
	Granular	1·529	1·533	1·343	1·350	1·436	1·442
III	Shredded	1·533	1·535	1·341	1·365	1·437	1·450
	Granular	1·532	1·533	1·342	1·350	1·437	1·442

Analysis of Variance (for III only)

Source of Variation	Mean Square			Environments Contrasted	
	Controlled Environment	Glasshouse	Environments Combined	Source	Mean Square
				Environment	4·042321***
Agar	0·000086	0·002926*	0·002005	Agar × environment	0·001007
Preplanting	0·000243	0·014790***	0·009407***	Preplanting × environment	0·005626**
Agar × preplanting	0·000012	0·003682*	0·002071	Agar × preplanting × environment	0·001623
Error	0·000830	0·000651	0·000740	Error	0·000740

*$P<0·05$. **$P<0·01$. ***$P<0·001$.

In view of the profound effect of the environment on the total number of nodules and on the dry weights of the plants, the statistical analysis was carried out on logarithms of counts so that the relative effects of the nature of the agar and preplanting could be compared in the two environments.

(*b*) *Time to Initial Nodulation*

Means for the two observers and the analysis are given in Table 2. The effect of preplanting in delaying nodulation is just significant for I ($P = 0·05$) but is not significant for II. For both observers the pooled higher interactions are larger than

the replicate variation within treatments, which may be because subjective rejection had removed valid plants from the tail region of the replicate frequency distribution. If, under these circumstances, the mean square for higher order interactions is regarded as a more satisfactory error term, no main effects are significant.

The mean time from sowing to initial nodulation for the whole experiment was 10·5 days.

(*c*) *Total Number of Nodules*

Means for I, II, and III, the discrepancy between I and II for statistically acceptable plants, and analyses of these data are set out in Table 3. For the experiment as a whole, preplanting did not significantly influence the total number of nodules formed, but effects arising from the observers, nature of the agar, and environment were highly significant.

TABLE 5

DEGREE OF AGREEMENT BETWEEN OBSERVERS IN REJECTION OF PLANTS

Observer	No. of Plants Rejected*	Observer	No. of Plants Rejected*	Observer	No. of Plants Rejected*
I, II, and III	16	I only	5	I	22
I and II only	0	II only	3	II	20
I and III only	1	III only	1	III	19
II and III only	1				

* One tube was preplanted by mistake and one apparently healthy plant failed to nodulate, so that 478 possibly acceptable plants remained.

Considering first the observers, the mean count for I is more than for II, the difference being significantly greater for plants grown in the glasshouse than for plants grown in the controlled environment. However, there is no interaction between the observer and effects due to either the nature of the agar or to preplanting.

In the controlled environment, preplanting caused a reduction (*c.* 9%) in the number of nodules on plants growing in the medium prepared from granular agar, but brought about an increase (*c.* 16%) in the number formed on plants growing in the medium prepared from shredded agar, this reversal being highly significant ($P<0·01$ for I; $P<0·001$ for II and III).

(*d*) *Dry Weights of Plants*

The means for I, II, and III are given in Table 4; as the observer effect through plant selection is clearly negligible the analysis in Table 4 relates to III only. Because the response to type of agar and to preplanting seems to be confined to plants grown in the glasshouse, both a separate analysis for each environment and a combined analysis are given in Table 4.

The large significant depression ($P<0·001$) in plant yield observed in the glasshouse was reflected in the appearance of the plants. Those grown in the controlled

environment had about seven dark green trifoliate leaves while most of those grown in the glasshouse bore only four pale green leaves. In the glasshouse, preplanting significantly depressed ($P<0\cdot001$) dry weights of plants. These effects do not appear to be related to the effects of preplanting and the nature of the agar medium on the number of nodules formed after 51 days.

IV. Discussion

The results show that precise data can be obtained by selecting uniform plants by statistically-acceptable methods and by standardizing the experimental technique; effects which were quite small in magnitude sometimes proved to be highly significant (Table 4).

It is clear also, from Tables 2 and 3, that the two independent observers agreed on the main effects which were found to be significant at the $P<0\cdot01$ level, despite the fact that in one instance (Table 3) their results differed in a highly significant manner ($P<0\cdot001$). However, there was some difference between observers' results concerning effects of low statistical significance ($P = 0\cdot05$, Table 2); consequently, in the following discussion effects will only be considered to be valid if significant at the $P<0\cdot01$ level.

The time interval between sowing and initial nodulation was independent of any of the treatments imposed on the plants, whereas several treatments affected the total number of nodules formed. Thus, it seems that different processes govern the initial and subsequent nodulation of *T. subterraneum* cv. Mount Barker growing under the conditions described.

The number of nodules formed during the period of the experiment was profoundly influenced by the environment and by the nature of the agar used to prepare the root media, but the effect of preplanting was small in magnitude and varied in a highly significant manner with the nature of the agar. The environmental conditions also had a highly significant effect on plant yield. This effect, when considered in relation to the number of nodules formed and the appearance of the plants may well reflect the efficiency of nitrogen fixation under the different conditions, and in particular, the unfavourably high temperature encountered in the glasshouse.

Further investigation of these interesting physiological differences is desirable.

The results recorded in Table 1 reveal that preplanting removes nitrogenous compounds from the root medium. The data in Table 3, relating to plants grown in the controlled environment, suggest that preplanting of the shredded agar medium may remove an inhibitor of nodulation but, on the other hand, the roots of the donor plant may exude an inhibitor of nodulation when grown on the medium prepared from granular agar. As preplanting leads to a slight reduction in the number of nodules formed only when plants are grown under optimal conditions (i.e. on granular agar in the controlled environment) and as this effect may be annulled by changing the cultural conditions, the role of root exudates derived from uninoculated donor plants in the nodulation of this species is considered to be of minor importance.

It is concluded that further studies on the growth and course of nodulation of *T. subterraneum* cv. Mount Barker should be undertaken before chemical fractionation of the root exudate of this species is warranted.

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