Plant and Soil XXV, no. 3 December 1966

STUDIES ON MICROBIAL ANTAGONISM IN THE ESTABLISHMENT OF CLOVER PASTURE

II. THE EFFECT OF SAPROPHYTIC SOIL FUNGI UPON *RHIZOBIUM TRIFOLII* AND THE GROWTH OF SUBTERRANEAN CLOVER

by A. A. HOLLAND* and C. A. PARKER

Department of Soil Science and Plant Nutrition Institute of Agriculture, The University of Western Australia, Nedlands, Western Australia

INTRODUCTION

In previous publications, Holland [6], described a problem of clover establishment on certain newly cleared sandy soils in Western Australia and presented the results of a microbiological investigation. The population of fungi in these soils showed marked changes in species composition following cultivation and establishment of subterranean clover *Trifolium subterraneum* L.

This paper reports the effect of saprophytic fungi isolated from both virgin sandy soils and established clover pastures on *Rhizobium trifolii* and on the nodulation and growth of subterranean clover.

MATERIALS AND METHODS

1. *Chlorophyll and anthocyanin*

The preparation of plant samples for chlorophyll determination was based on the method described by Arnon [1] and the quantity of chlorophyll was estimated by the procedure of Mackinney [10]. Anthocyanin was determined by the method employed by Swain and Hillis [13].

---

* Present address: Botany Department, Monash University, Melbourne, Australia.

2. *Bioassay of antibiotics from micro-organisms, soil and plant extracts*

Six replicate cultures of each micro-organism isolated from virgin soils and established pastures [8], were grown in 25 ml of broth [9] in agitated culture at 25°C for 14 days. Two ml of broth were drawn off each day from the third to the fourteenth day, asceptically filtered [16] and tested for antibiotics using the 'hole plate' method of Vesterdal [14]. Test and control broths were placed in holes 5 mm in diameter cut in yeast mannitol agar [3] seeded with a 48-hour culture of *R. trifolii*. The plates were incubated at 25°C and examined after 12, 24, and 48 hours of growth. Inhibition zones were measured and graded as bactericidal or bacteriostatic following Marshall and Hrenoff [11]. Extracts from soil, sand culture and crushed plant material were also filtered and tested for antibiotics using the methods already outlined.

3. *Seed inoculation*

The clover seed was inoculated with a pure culture of *R. trifolii* so as to give an initial number of 500 to 1000 viable bacteria per seed.

4. *Soil sterilisation*

Soil samples were sterilized by autoclaving at 15 lb/sq. inch for one hour.

5. *Seed sterilization*

The clover seed was graded to a uniform size and sterilized using a mixture of equal volumes of 30 volumes per cent hydrogen peroxide and absolute alcohol (Harris, J. R., priv. com.).

6. *Nutrient solution*

The basic nutrient solution for the clover plants was that of Rovira [12] with the addition of a trace element solution [5]. When a carbon supplement was required to stimulate fungal growth 20 g of sucrose was added to each litre of nutrient solution or 4 per cent ($^{w}/_{w}$) sterilized wheaten straw was mixed with the sand.

For the aseptic tube culture of subterranean clover plants nutrient solution was added to the tubes to bring the sterilized oven dry sand to 60 per cent of the water holding capacity of the sand. It was usually not necessary to add more solution as plant growth in the tubes was not taken past the third trifoliate leaf stage. Clover plants were grown in the field with zinc-copper-superphosphate fertilizer at the rate of 180 pounds per acre. The fertilizer contained 1.0 per cent copper as copper ore, 1.15 per cent zinc as zinc oxide, 12.75 per cent water soluble phosphoric acid, 2.25 per cent citrate soluble phosphoric acid and 3.5 per cent acid soluble phosphoric acid.

6. *Temperature and light*

Clover plants were grown in the laboratory at a constant temperature of 15°C under fluorescent lights.

7. *Vegetative activity of the fungal cultures grown in conjunction with subterranean clover*

The method described by Warcup [15] was used to estimate the hyphal activity of fungi in the soils under test.

8. *Counts of the rhizobia in the rhizosphere of subterranean clover*

The method used to determine the number of root-nodule bacteria in the rhizosphere of subterranean clover plants was similar to that employed by Hely, Bergersen and Brockwell [4].

EXPERIMENTAL RESULTS

1. *Symptoms associated with nodulation failure in the field*

The inoculated seed was sown about 2 cm deep in the newly cleared soil in the field. Germination and early growth were good but stunting and discolouration of the cotyledons, petioles and leaves were apparent within three weeks of emergence. The foliage developed a distinctive purplish-red colour, which was associated with a reduction in chlorophyll and an increase in anthocyanin content (see Table 1).

TABLE 1

The chlorophyll and relative anthocyanin content of clover leaf and petiole material from (i) normal plants from an established pasture and (ii) plants showing the purplish-red foliage associated with nodulation failure

Plant Material	mg of chlorophyll in 1.0 g of fresh plant material	Relative anthocyanin content
(i) Normal trifoliate leaf	4.2	10
(i) Normal petiole	0.52	8
(ii) Trifoliate leaf	0.36	510
(ii) Petiole from a purplish red leaf	0.18	3040
(ii) Trifoliate leaf red	0.18	785
(ii) Petiole from a red leaf	0.08	3340

The stunting of the clover plants grown in newly cleared areas is illustrated in Table 2, which compares the growth made on untreated soil with that made when the soil was chemically sterilized with Vapam (sodium N-methyldithiocarbamate). The sterilization procedure was that employed by Cass Smith and Holland [2].

TABLE 2

Oven-dry weights of subterranean clover plants grown in the field on chemically sterilized and unsterilized soil at Wongan Hills (four monts after seeding)

	Sterilized soil	Un-sterilized soil	Level of signifi-cance
Number of plants	42	43	
Number of nodulated plants	42	4	$P < 0.1\%$
Mean foliage weight (mg/plant)	463	90	$P < 0.1\%$
Mean root weight (mg/plant)	206	76	$P < 0.1\%$

2. *The number of rhizobia in the rhizosphere*

Serial dilutions from the rhizosphere of young seedlings grown in the field in chemically sterilized and unsterilized soil indicated that the mean probable number of root-nodule bacteria was greater than 10^3 bacteria per root system in the sterilized soil, whereas none was detected on the roots of plants grown in unsterilized soil.

3. *The detection of antibiotics in soil from newly cleared areas*

Aqueous extracts from the problem soils twice showed the presence of antibiotics early in the growing season. The greatest inhibition zone observed on seeded agar was 33 mm in diameter. Attempts to concentrate the inhibitors failed due to rapid loss of activity. Aqueous extracts from similar soils at Wongan Hills which now support established clover pastures, as well as extracts from non problem soils supporting clover pastures have not produced inhibition zones.

Extracts from crushed clover leaves which showed the purplish-red foliage associated with nodulation failure produced bacteriostatic inhibition zones surrounded by stimulation zones. The inhibition zones were present even when the soil extracts gave no indication of the presence of inhitors. This may have been due to absorption of antibiotics by the root system and translocation to the leaves where they were concentrated sufficiently to be detected. Extracts from healthy clover leaves did not produce inhibition zones. Zones of bacterial stimulation were observed with extracts from healthy green leaves and from purplish-red leaves beyond the zone of inhibition.

4. *Antibiotic production in pure culture*

Fifty-seven of the 286 micro-organisms tested synthesized antibiotics inhibiting the growth of *R. trifolii* (Table 3). The genus *Penicillium* contained the greatest of species and strains producing

TABLE 3

The antibiotic activity towards *Rhizobium trifolii* of micro-organisms isolated from newly cleared soils and soils under established clover pastures

Micro-organisms isolated	Newly cleared soils			Established clover soils		
	Number tested	Anitibiotic production		Number tested	Antibiotic production	
		Bacteri cidal	Bacteri ostatic		Bacteri cidal	Bacteri ostatic
Rhizopus	—	—	—	2	0	0
Mucor	—	—	—	4	0	0
Mortierella	8	0	0	5	0	0
Non-sporing phycomycetes	2	2	0	—	—	—
Ascomycetes	4	0	0	5	0	1
Phoma	5	0	1	5	0	0
Macrophomina	—	—	—	1	0	0
Monilia	—	—	—	1	0	0
Cephalosporium	—	—	—	1	0	0
Trichoderma	2	0	0	6	0	0
Aspergillus	7	0	2	8	0	2
Penicillium	47	17	17	19	1	4
Sporotrichum	—	—	—	1	0	0
Gliocladium	1	0	0	—	—	—
Arthrobotrys	—	—	—	1	0	0
Verticillium	—	—	—	1	0	0
Pullularia	—	—	—	1	0	0
Trichosporium	—	—	—	1	0	0
Cladosporium	—	—	—	1	0	0
Curvularia	11	0	0	7	0	0
Helmintho-sporium	—	—	—	1	0	0
Stemphylium	—	—	—	6	0	0
Alternaria	—	—	—	3	0	0
Heterobotrys	—	—	—	1	0	0
Fusarium	4	0	0	9	0	0
Rhizoctonia	1	0	0	1	0	0
Pellicularia	—	—	—	1	0	0
Sclerotium	4	0	0	2	0	0
Non-sporing cultures	12	0	0	1	0	0
Actinomycetes	44	0	5	12	0	3
Bacterial cultures	15	0	1	12	0	1
Total	167	19	26	119	1	11

antibiotics as well as the most toxic antibiotics, whereas most of the other cultures of fungi, actinomycetes and bacteria did not produce inhibitors. Twenty-seven per cent of micro-organisms isolated from newly cleared soils proved capable of synthesizing antibiotics but only ten per cent from established pastures produced antibiotics.

5. *The effect of single cultures of micro-organisms on clover seedlings and root-nodule bacteria*

Cultures of 260 species and strains of micro-organisms isolated from soil from both newly cleared sites and established clover pastures were grown aseptically in tubes of sterilized sand in

TABLE 4

The effect of carbon supplements upon antibiotic production by micro-organisms and upon the nodulation and yield of clover seedlings grown in the presence of these micro-organisms

Nutrient * medium	Micro-organisms	Antibiotic production against *R. trifolii*	Nodulation	Mean foliage weight (mg d.b)	Mean root weight (mg d.b)
1	Control	−	+	15	5
	Neutral **	−	+	17	4
	Antagonistic †	−	+	15	5
2	Control	−	+	14	5
	Neutral	−	+	14	4
	Antagonistic	−	+	13	5
3	Control	−	+	17	5
	Neutral	−	+	15	5
	Antagonistic	+	−	3	3
4	Control	−	+	15	5
	Neutral	−	+	14	4
	Antagonistic	+	−	8	4
5	Control	−	+	14	5
	Neutral	−	+	15	5
	Antagonistic	+	−	4	3
6	Control	−	+	14	5
	Neutral	−	+	13	4
	Antagonistic	+	−	9	5

* 1 = Complete nutrient solution
2 = Nutrient solution minus nitrogen
3 = Complete nutrient solution plus 2 per cent sucrose
4 = Nutrient solution minus nitrogen plus 2 per cent sucrose
5 = Complete nutrient solution plus 4 per cent straw
6 = Nutrient solution minus nitrogen plus 4 per cent straw

** 229 micro-organisms detailed in text

† 31 micro-organisms detailed in text

conjunction with inoculated subterranean clover. Extracts were obtained from the sand in each tube and tested for the presence of antibiotics inhibitory to *R. trifolii*.

The results are presented in Table 4 and have been grouped as follows:

(i) controls containing no micro-organisms.

(ii) neutral micro-organisms which did not inhibit nodulation or plant growth. These numbered 229 and consisted of species of the following:

2 Rhizopus, 4 Mucor, 11 Mortierella, 9 Ascomycetes, 10 Phoma, 1 Macrophomina, 8 Trichoderma, 12 Aspergillus, 32 Penicillium (species and strains) 1 Sporotrichum, 1 Gliocladium, 1 Arthrobotrys, 1 Verticillium, 1 Pullularia, 1 Trichosporium, 1 Cladosporium, 17 Curvularia (species and strains) 6 Stemphylium, 1 Helminthosporium, 2 Alternaria, 11 Fusarium, 6 Sclerotium, 13 non-sporing cultures, 49 actinomycetes, and 27 bacterial cultures,

(iii) antagonistic micro-organisms which consisted of 2 non-sporing Phycomycetes and 29 species and strains of Penicillium.

Nodulation and growth of the control plants grown with rhizobia only were not affected by the addition of sucrose, straw or nitrogen (Table 4). The micro-organisms grouped as "neutral" did not produce detectable antibiotic(s) in sand culture with either sucrose or straw, nor did they inhibit nodulation. The leaves of the clover seedlings were a normal green in colour and the seedlings did not differ in foliage or root weights from the control plants.

The micro-organisms grouped as antagonistic produced antibiotics only where sucrose and straw amendments had been used; under these conditions foliage and root weights of the clover seedlings were reduced and nodulation was inhibited (see Table 4). Furthermore, a purplish-red colour similar to that displayed by non-nodulated clover plants in the field, frequently developed in the foliage of the seedlings.

All the micro-organisms that either synthesized antibiotics in sand culture, inhibited nodulation, or produced phytotoxic substances, were originally isolated from virgin soils. All the micro-organisms isolated from soil under established clover pastures and most from virgin soils proved to be neutral. Microscopic examination indicated that the antagonistic organisms failed to grow, either in

soil or in the rhizosphere, unless stimulated by the addition of sucrose or straw.

It is worthy of note that not all the organisms which produced antibiotic in broth culture (table 3) inhibited nodulation in sand culture. Every organism, however, which inhibited nodulation and depressed seedling growth also produced detectable antibiotic in broth culture.

5. *The effect upon nodulation and growth of clover seedlings of groups of micro-organisms from virgin and pasture soils*

Sand containing 4 per cent (w/w) sterilized macerated straw was placed in seed boxes and inoculated with two groups of micro-organisms, one from virgin soils and one from established clover pastures. Because of the difficulty of assessing the hyphal activity of the micro-organisms after their inoculation into the straw enriched sand, only those organisms were used which were most frequently isolated from each soil. Those from virgin soils numbered 79 and consisted of species of: 2 non-sporing Phycomycetes, 8 Mortierella, 3 Phoma, 1 Trichoderma, 4 Aspergillus, 15 Penicillium, 9 Curvularia, 4 Fusarium, 4 Sclerotium, 12 non-sporing cultures and 17 actinomycete cultures.

Those from established clover pastures numbered 49 and consisted of: 4 Mortierella, 2 Rhizopus, 4 Mucor, 1 Phoma, 1 Macrophomina, 1 Trichoderma, 3 Aspergillus, 2 Curvularia, 5 Penicillium, 8 Fusarium, 1 Sporotrichum, 1 Pullularia, 1 Cladosporium, 1 Stemphylium, 1 Sclerotium, 1 non-sporing culture and 12 actinomycete cultures.

The box cultures were watered with the nitrogen-free plant nutrient solution and then held at 15°C in growth cabinets for 7 days before inoculated clover seeds were planted.

Nodulation was inhibited in the presence of micro-organisms originally isolated from newly cleared soils. Moreover the clover leaves developed reddish discolouration similar to though not as well defined as, that associated with nodulation failure in the field or with that found in plants grown with certain pure cultures of micro-organisms in the test tubes. There was a decrease in the foliage weights but no significant difference in the root weights of the clover seedlings when grown with the two different groups of micro-organisms (see Table 5).

TABLE 5

The nodulation and growth of clover seedlings grown in straw enriched sand and inoculated with micro-organisms isolated from virgin soils and similar soils supporting clover pastures

	Micro-organisms from newly cleared soils	Micro-organisms from established pastures	Level of significance
Number of plants	50	50	
Number nodulated	3	50	$P < 0.1\%$
Mean fresh foliage weight (mg)	162	210	$P < 0.1\%$
Mean fresh root weight (mg)	54	59	Not significant

Soil water extracted 6 days after inoculation with the micro-organisms isolated from virgin soils was toxic to *R. trifolii* whereas antibiotics were not detected in extracts from the soil inoculated with micro-organisms isolated from soils under established clover pastures.

Species of Mortierella, Penicillium, Curvularia, Sclerotium and non-sporing Phycomycetes were found to be vegetatively active on the straw inclusions in the soil inoculated with micro-organisms originally isolated from virgin soil, whilst species of Mortierella, Rhizopus, Mucor, Macrophomina, Aspergillus, Fusarium, and Sclerotium were active in the soil inoculated with those micro-organisms isolated from soils under established clover pastures.

These results again indicate that certain micro-organisms originally present in virgin soil can produce substances which are toxic both to *R. trifolii* and to the host plants.

DISCUSSION

The nodulation failure of subterranean clover on newly cleared lateritic podzolic soils has been shown to be overcome by moist heat [7], chemical sterilization or ageing the soils before planting the inoculated clover seed. Generally soil sterilization is accompanied by a release of nutrients and therefore the improvement of clover nodulation in sterilized soils could be attributed to this rather than to the destruction of the endemic soil flora. However, Shier and

Dunne (cited from Cass Smith and Holland [2]) found no improvement in nodulation using a wide range of both major and trace elements.

The micro-organisms tested in this investigation were found, in the main, to be compatible with the clover plant and its associated root-nodule bacteria. The exceptions were certain antibiotic producing fungi, especially in the genus Penicillium, which inhibited root-nodule bacteria and growth of seedlings.

Antibiotics were detected only twice in newly cleared field soils during this investigation but were frequently detected in extracts of seedlings grown on problem soils.

The failure to detect them more often in soil, particularly when micro-organisms known to be capable of producing antibiotics were present in large numbers, may possibly be attributed to technique. However, clover plants growing in such soil appeared to have concentrated the antibiotic in their leaves to a point where it was detectable in the agar-plate bioassay.

In this investigation certain species of Penicillium and non-sporing phycomycetes both in pure and in mixed cultures synthesized substances that inhibited nodulation and were phytotoxic, resulting in a stunting of the plant and decreased chlorophyll and increased anthocyanin in the foliage. These plants showed the same symptoms displayed by the field grown plants.

A comparison of the microbial populations in a problem soil and in soils where clover has become established shows that there has been a major shift in the fungal species [6 7 8]. A substantial number of fungi isolated from newly cleared soil have produced antibiotics which are toxic to both nodule bacteria and clover plants. These fungi flourish on the organic debris remaining in the soil when the original vegetation is cleared [6 7 8]. As the soil ages the population changes [8] and becomes mainly compatible with rhizobia and with the clover seedlings. Clover pastures may then be established.

We conclude from the evidence presented that the problem of clover establishment on newly cleared soils at Wongan Hills is due to microbial antagonism.

SUMMARY

In certain newly cleared soils in Western Australia subterranean clover fails to nodulate. The plants are stunted and discoloured with greatly in-

creased anthocyanin and decreased chlorophyll in the leaves and petioles. Chemical sterilization of such a soil in the field permitted nodulation and normal growth [2].

Water extracts from the problem soils were twice proved toxic to *R. trifolii*, and extracts from the leaves of affected plants were frequently so. In sand culture experiments, where inoculated clover seedlings were grown together with single strains of soil isolates, antibiotics were produced by some isolates but only when sucrose or straw was added to the sand. Thirty-one fungi produced detectable antibiotic(s) and inhibited nodulation and growth of the seedlings. None of the remaining 153 fungal, 49 actinomycete or 27 bacterial cultures affected the seedlings. The addition to straw enriched sand of mixed cultures made up of the most frequently occuring micro-organisms from. virgin soil in one group, and from soil under established pasture in another, showed those from virgin soils exerting a marked depression upon nodulation and growth of clover seedlings.

Seedlings grown together with single or mixed cultures of antibiotic-producing organisms frequently displayed symptoms of stunting and discolouration typical of the field syndrome.

The problem of clover establishment on virgin soils appears to be caused by antibiotic-producing fungi which proliferate on the organic debris remaining after the original vegetation has been removed.

ACKNOWLEDGMENTS

We wish to express our appreciation to Mr. C. A. P. Boundy, of the Division of Mathematical Statistics of C.S.I.R.O. for statistical analysis of the data, and to the Committee of the Rural Credits Development Fund of the Reserve Bank of Australia for financial support.

Received July 12, 1965 Revised June 3, 1966

REFERENCES

1 Arnon, D. I., Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. **24**, 1–15 (1949).

2 Cass Smith, W. P. and Holland, A. A., The effect of soil fungicides and fumigants on the growth of subterranean clover on new light land. J. Agr. W. Australia **7**, (3rd Series) 225–231 (1958).

3 Fred, E. B., Baldwin, I. L. and McCoy, E., Root-nodule Bacteria and Leguminous Plants. Univ. Wisconsin Press (1932).

4 Hely, F. W., Bergersen, F. J. and Brockwell, J., Microbial antagonism in the rhizosphere as a factor in the failure of inoculation of subterranean clover. Australian J. Agr. Research **8**, 24–44 (1957).

5 Hoagland, D. R. and Arnon, D.I., The water culture method for growing plants without soil. Univ. Calif. Agr. Exp. Sta. Circ. **347** (1938).

6 Holland, A. A., The effects of indigenous saprophytic fungi upon nodulation and establishment of subterranean clover. *In*: Antibiotics in Agriculture. p. 147–164. Proc. 9th Easter School in Agriculture, Nottingham, Ed. M. Woodbine, Butterworths London (1962).
7 Holland, A. A., Microbial influences in pasture establishment. Ph. D. Thesis. University of Western Australia (1963).
8 Holland, A. A., Studies on microbial antagonism in the establishment of clover pasture. 1. The number of species and frequency of occurrence of the major fungi. Plant and Soil **25**, 238–248 (1966).
9 Lacey, Margaret S., The antibiotic properties of fifty-two strains of *Fusarium*. J. Gen. Microbiol. **4**, 122–131 (1950).
10 Mackinney, G., Absorption of light by chlorophyll solutions. J. Biol. Chem. **140**, 315–322 (1941).
11 Marshall, N. S. and Hrenoff, A. C., Bacteriostasis. J. Infect. Diseases **61**, 42–54 (1937).
12 Rovira, A. D., Plant root excretions in relation to the rhizosphere effect. Plant and Soil **7**, 178–217 (1956).
13 Swain, T. and Hillis, W. E., The quantitative analysis of phenolic constituents. J. Sci. Food Agr. **10**, 63–68 (1959).
14 Vesterdal, J., The agar cup method for the estimation of Penicillin. Acta Pharmacol. Toxocol. **2**, 9–21 (1946).
15 Warcup, J. H., Isolation of fungi from hyphae present in soil. Nature, Lond. **175**, 953 (1955).
16 Wyllie, D. M., Filtration by centrifugal force. Pharmacol. J. **175**, 492. (1955).