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INOCULANTS AND ECOLOGY OF RHIZOBIUM

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Abstract

The natural and modified environments encountered by Rhizobium are discussed with particular attention to the concept of competitive ability and recognition methods. Definition of the need for inoculation is discussed, some pertinent questions put, and the relevance of inoculant quality emphasised.

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The natural environment

Rhizobium is a saprophyte and a facultative symbiont, so that the range of environments encountered is wide.

The natural ecological "niches" may be subdivided into (a) the soil which is subject to a declining influence of the host crop with time, and changes in associated microflora, (b) the rhizosphere, where the qualitative diversity of the microbial population may be reduced but populations of particular organisms are higher, possibly with more direct competition for the same metabolites excreted by the root and (c) the symbiotic environment which covers infection to nodulation where the complexities of the compatibility with host are the overriding environmental influences.

The modified environment

Rhizobium as a saprophyte

Man can affect the environment in many ways even with a traditional crop. Cultivation of soil results in changes in nutritional status, moisture loss, temperature changes etc. which directly influence the persistence of the existing rhizobia and the associated saprophytic soil microflora. In many soils it is reasonable to expect survival and persistance except in soils subject to flooding where it may become a critical issue (e.g. paddy, Toomsan *et al*[1] this conference).

Little attention is given to the distribution of rhizobia among the various soil fractions (Parker et al, 1977)[2]. We are not in a position to explain the seasonal variations found in Rhizobium populations (e.g. Brockwell, 1963)[3] nor the variable vertical distributions found with pigeonpea and chickpea at ICRISAT (Kumar Rao and Rupela personal communication). Nodules can also be absent at depths where rhizobia are known to be present.

The modifications imposed by man are generally due to introduction of new crops, and thus the importance of the rhizosphere is paramount. Although evidence points to the legume rhizosphere as being favourable for rhizobia there is diversity of opinion as to whether the stimulation is greatest for the rhizobia infective on that legume.Moreover exceptions occur in one case, the populations on R. trifolii found after sowing into a Rhizobium-free field with a range of hosts were greatest where the rhizobia were introduced with wheat rather than clover plants (unpublished data). In some extreme circumstances survival in the field between seasons can be at risk because Rhizobium does not survive once the rhizosphere had disappeared (the second year mortality problem in Western Australia as reported by Marshall et al, 1963)[4].

7. K.G. Gupta and A. Sen, Indian J. Agric. Sci. 32, 268 (1962).

8. T. Matsumoto, Y. Yamamoto and M. Yatazawa, Jour. Soil and Manure, Japan 47, 463 (1976).

9. I. Smith, Chromatographic techniques, William Heinemann Medical Book Ltd. London (1958).

10. F. Trijbels and Vogels, Biochem. Biophys. Acta, 113, 292 (1966).

11. R. Borchers, Analytical Biochemistry, 79, 612 (1974).

12. V. Ranga Rao, S. Sopory and N.S. Subba Rao, Curr. Sci. 43, 503 (1974)

13. V. Ranga Rao and N.S. Subba Rao, Zeitzshrift fur Pflazenphysiol., 80, 14 (1976).

14. H.J. Evans, D.W. Emerick, T. Ruiz - Arguese, R.J. Maier and S.L. Albrecht, In Nitrogen Fixation volume II, Ed. W.E. Newton and W.H. Orne and Johnson, 69 (1980).

15. R.K. Dadarwal, Shashi Prabha and P. Taure, Curr. Sci. 48, 647 (1979).

16. N.S. Subba Rao, Annual Report of All India Coordinated Project of Biological Nitrogen Fixation, ICAR, (1981).

17. K. Iswaran, W.V.B. Sundara Rao, S.P. Magu and K.S. Jauhri, Curr. Sci. 1, 468 (1969).

18. R. Kandaswamy and N.N. Prasad, Curr. Sci. 40, 496 (1971).

19. K.V.B.R. Tilak and N.S. Subba Rao, Fert. News (India) 23, 25 (1978).

procedure works - in fact the presence of nodules high on the crown of some plants ("crown" nodulation) often is interpreted as successful establishment of the inoculum.

Alternative inoculation methods are necessary when chemicals toxic to rhizobia have been used as dusts or for species such as groundnut and soybean which are susceptible to damage when seeds are inoculated with any liquid adhesive. The normal carrier-based inocula can be successfully applied separately from the seed as suggested by Bonnier 1960[8]. Liquid suspensions applied to soil are favoured in situations where labour for inoculation is expensive but machinery can be adapted to carry and dispense the liquid. Data by Brockwell et al (1980)[9] showed that while all methods were successful under favourable conditions, "liquid" and "solid" methods were superior to seed inoculation under adverse conditions. Another modification to technology involves use of large granules of peat inoculant (ca. 1 mm diam) which has sufficient flow characteristics and is applied in sufficient quantity to be distributed from a separate box on sowing machinery.

In addition to cultivation effects, the environment may also be modified by fertilizers. In Australian low-P soils, when pasture legumes are commonly sown, superphosphate is used and contact with the acidic fertilizer can certainly be harmful. Often the soils themselves are acidic and lime coating of seed has been a popular measure for additional protection.

With all techniques we hope to provide the rhizobia with a competitive advantage over the soil population by suitable placement and high numbers.

Rhizobium as a symbiont

Our ultimate aim with inoculation is to provide the host with the best symbiont. The suitable rhizobia have been variously described as virulent, incursive and effective (the VIE strains of Harris, 1954)[10] symbiotically competent (Bromfield and Ayanaba, 1980)[11] and competitive. Strains most frequently nodulating the host from a mixed population are described as competitive. We are as yet unable to ascribe any intrinsic "competitive" character to Rhizobium itself although such characters as bacteriocin production could be involved.

inherent character of Rhizobium even with single strain inoculants.

Recognition and identification of rhizobia

Serology has received most consistent attention sinc it was popularized by Vincent (1941)[15]. The original te nique involved simple agglutination. In relation to other techniques it is slow, less sensitive and is most easily conducted with pure cultures although squashes of larger nodules such as those of soybean are readily tested (Means, Johnson and Date, 1964)[16].

The immunodiffusion test is the simplest serological test to set up (Schwinghamer and Dudman, 1980)[17] and allows direct comparison of various antigens. Squashes of larger nodules can also be used.

The fluorescent antibody technique can be used for identification of a very few cells and direct observation of squashes of very small nodules is practicable. The technique was popularized by Trinick (1969)[18]. Conjugated antibodies are stable and can be used economically (Schinghamer and Dudman,1980)[17]. Bohlool and Schmidt (1970)[19] have successfully applied the technique directly to soil suspensions for examination of populations living saprophytically. The indirect method using anti-rabbit antiserum is easier since conjugation of each strain antiserum is not necessary.

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The serological technique most recently applied to Rhizobium is that of enzyme-linked immunosorbent assay (ELISA) which involves, in direct (Kishinevsky and Bar-Joseph, 1978)[20] and indirect (Berger et al, 1979)[21] applications, binding of specific enzyme-linked antibody to the antigen with subsequent addition of a suitable colourless substrate for the enzyme, which then converts it to a coloured product. The test is extremely sensitive and it can be totally automated and 1500 samples can be tested in a day.

Antibiotic resistance has been utilized in two ways. Some workers (eg. Obaton, 1971[22]; Jones and Bromfield, 1978)[23] have used mutants selected against high concentrations of antibiotics (generally between 150-500 µg/ml) and recoverable in antibiotic medium. Vidor and Miller (1980)[24] and Bushby (1981)[25] have used the technique for recognition of cultures from soils and rhizosphere. Srivastava et al (1980)[26] also used mutants resistant to chlorate, azide, erythromycin and phage sensitivity.

Recently Beynon and Josey (1980)[27] have made use of inherent resistance to antibiotics at low levels (e.g. 1 and 10 µg/ml). As well as differentiation of the inoculant strains, the technique also separates members of unknown population. They found 54 different

resistance patterns in one field of Phaseolus vulgaris. Unfortunately subsequent use of the technique for examination of isolation from chickpea and pigeonpea at ICRISAT have not been rewarding (Stein and Bromfield, personal communication). Other methods such as electrophorosis (Noel and Brill, 1980)[28] have also proved successful although they may be less practical than serological tests.

The need for inoculation.

To sow a legume in a new environment we must ask:

1) will the legumes nodulate naturally ?
2) is the nodulation optimum for the particular situation ?
3) can it be improved by inoculation with introduced strains ?

A few authors have addressed this question by designing standard experiments for use in a range of environments (e.g. Nutman, 1976[29]; Date, 1977[30]; Date and Halliday, 1980)[31]. The treatments in the NifTAL Project (1979)[32] include inoculated, uninoculated and N treatments with "adequate" and "farmers level" fertilizers. Such studies only require a Rhizobium inoculant known to be effective on the host.

It is surprising how rarely such approaches are adopted. We all too readily assume that inoculation is a

desirable procedure and therefore is always justified. In advocating use of such simple experimentation let us not overlook the need for additional useful tests e.g. an estimate of the number of rhizobia in the natural soil and serological identification.

Ahmad et al (1981)[33] examined 4 sites with low soil N levels in Nigeria by sowing a wide range of cowpea germplasm without inoculation, in the presence and absence of N. Their objective was to examine the diversity of Rhizobium strains naturally present. The experiment provided a basic collection of Rhizobium for examination but while allowing some judgement of the need for inoculation did not provide a suitable control treatment.

The difficulty of interpretation of incomplete studies is illustrated in some of our own studies from ICRISAT in 1981. We provided inoculated and uninoculated seeds of pigeonpea to farmers who sowed them in alternating blocks. In one field of 6 we found a significant response in nodulation at 35 days. However growth of some crops was relatively poor overall - without an N treatment and without a full nutrient treatment we cannot successfully answer the questions raised. Observations without experimentation such as in field surveys of nodulation have limitations. Lack of nodulation does not necessarily mean that a response to inoculation will occur, but we are tempted to expect that it will.

Conclusions

There is a risk of overemphasis on particular aspects of Rhizobium studies, while so many ecological questions remain unanswered. Two aspects readily spring to mind, (a) strain collection and (b) field strain testing. Both activities, so essential to making a full use of our opportunities, can readily become ends in themselves, and can tie up resources and time which can be used more effectively in studying the field situation. Let us think seriously about informed selection of test sites, about the suitable reallocation of resources often available within other parts of our own institutions. Finally many important questions remain unanswered.

1) do farmers really need to inoculate ?
2) are soil populations of rhizobia adequate for new varieties ?
3) do we have reliable inocula when required ?
4) are the inoculant strains chosen for desirable attributes e.g. "competitive ability" measured in relevant environments ?
5) do we really have Rhizobium host x strain interactions ?
6) why do we find yield responses to strains isolated from local environments ? Are they inherently more suited to the environment ?
7) Are research station data relevant to the farmers' fields ?
8) What are the relative host and Rhizobium requirements in stress situations common in India e.g. saline soil, Zn & Fe deficiency ?
9) What is the place of alternative [illegible]

We need scientific answers to microbiological questions.

The quality of inoculants: I have left the discussion of the basic input in inoculation until last - viz. the inoculant itself. Unless we use and establish an inoculant with adequate rhizobia of the right type we are wasting our time and resources.

The definition of Rhizobium is that it nodulates a relevant legume. In this era of microbial genetics we have many variants including non-infective mutants but for the applied worker nodulation is the criterion. There are many gram negative rods in nature and there are many bacterial contaminants which look like Rhizobium - are they ? The history of inoculant manufacture and of many strain collections is full of examples of organisms which look like rhizobia and are not ! They helped to contribute to the problems which placed the inoculant industry of Australia in peril in the early 50's. Many inocula of poor quality were being sold and the losses of sowings of new legumes into poor soils were enormous.

The subsequent recovery of the Australia Inoculant industry (see Date, 1969)[34] and the reason for its high standing today, was due to application of sound microbiological techniques for evaluation of the inocula during and after production, and the acceptance by manufacturers of rejection of poor batches. The control measures remain time-consuming (plant dilution counts require at least 3 weeks after test for a reliable answer) and require some capital investment (for growth of plants in controlled conditions) and success of the quality control process requires absolute

technical or monetary commitment by an involved individual.

The Indian inoculant industry is in many ways where the Australian industry was in the 50's. We have examined local Indian inocula from many sources. Irrespective of private or public institution, origin the majority fail to pass the published standards of the Indian Standards Institution (1977)[35] when the number of rhizobia are estimated by the plant dilution infection count. Can we ensure that the Indian industry capitalizes on the known mistakes and recovers in time ? How many manufacturers have calculated the inoculum provided per seed by 10^6 rhizobia/gram when the ISI (1977) standard requires 10^8/gram carrier ?

Let us heed the title of a recent paper by Scudder (1978)[36] "Dead bacteria fix no N".

References

1. B. Toomsan, O.P. Rupela and P.J. Dart, Natl. Symp. on Biol. Nitr. Fixation, New Delhi, Feb. 25-27 1982 (1982)

2. C.A. Parker, M.J. Trinick, and D.L. Chatel, Chapter 9 in "A Treatise on Dinitrogen Fixation IV. Agronomy and Ecology" (Eds. R.W.F. Hardy and A.H. Gibson, John Wiley & Sons, pp.311-352) (1977)

3. J. Brockwell, CSIRO Divn. of Pl. Ind. Field Stat, Rec. 2:59-70 (196

4. K.C. Marshall, M.J. Mulcahy and M.S. Chowdhury, J. Aust. Inst. Agric. Sci. 29:160-164 (1963)

5. D.L. Chatel, R.W. Greenwood and C.A. Parker, 9th Int. Cong. Soil Sci. Vol 2, pp.65-73 (1968)

6. J. Brockwell, Aust. J. Agric. Res., 13:638 (1962)

7. J. Brockwell and F.W. Hely, Aust. J. Agric. Res., 13:1041-1053 (196

9. J. Brockwell, R.R. Gault, D.L. Chase, F.W. Hely, M. Ziron and E.J. Corbin, Aust. J. Agric. Res. 31:47-60 (1980)

10. J.R. Harris, Aust. J. Agric. Res., 5:247-270 (1954).

11. E.S.P. Bromfield and A. Ayanaba, Plant Soil 54:95-106 (1980)

12. G. Hardarson and D.G. Jones, Ann. Appl. Biol., 92:329-336 (1979)

13. A.C. Robinson, Aust. J. Agric. Res., 20:827-841 (1969)

14. R.A. Date and J. Brockwell, Chapter 14 in "Plant relations in pastures" (Ed. J.R. Wilson) CSIRO, East Melbourne, Victoria, (1978)

15. J.M. Vincent, Proc. Linn. Soc., NSW 67:145-154 (1941)

16. U.M. Means, H.W. Johnson and R.A. Date, J. Bact. 87:547 (1964)

17. Schwinghamer and Dudman, in "Methods for evaluating Biological Nitrogen Fixation" (Ed. F.J. Bergersen) John Wiley & Sons (1980)

18. M.J. Trinick, J. Appl. Bact., 32:181-186 (1969)

19. B.B. Bohlool and E.L. Schmidt, Soil Sci., 110:229-236 (1970)

20. B. Kishinevsky and M. Bar-Joseph, Can. J. Microbiol. 24:1537-1543 (1978)

21. J.A. Berger, S.N. May, L.R. Berger and B.B. Bohlool, Appl. Env. Microbiol., 37:642-646 (1979)

22. M.J. Obaton, C.R. Acad. Sci (Paris) 272:2630-2633 (1971)

23. D.G. Jones and E.S.P. Bromfield, Ann. Appl. Biol. 88:448-450 (1978)

24. C. Vidor and R.S. Miller, Soil Biol. Biochem., 12:483-487 (1980)

25. H.V.A. Bushby, in "Current perspectives in nitrogen fixation" (Ed. A.H. Gibson and W.E. Newton) Aust. Acad. Sci., Canberra p. 432 (1981)

26. B.S. Srivastava, B.K. Murthy, B.D. Singh, R.B. Singh, R.M. Singh, and B. Dhar, Beitr. Biol. Pflanzen, 55:147-155 (1980)

27. J.L. Beynon and D.P. Josey, J. Gen. Micro., 118:437-442 (1980)

28. K.D. Noel and W.J. Brill, Appl. Env. Microbiol., 48:931-938 (1980)

29. P.S. Nutman in "Symbiotic nitrogen fixation in plants" (Ed. P.S. Nutman) Cambridge University press, pp.211-237 (1976)

30. R.A. Date in "Exploiting the legume-Rhizobium symbiosis in tropical agriculture" (Ed. J.M. Vincent, A.S. Whitney and J. Bose) Coll. Trop. Agric. Misc. Publ. 145, University of Hawaii (197

31. R.A. Date and J. Halliday in "Advances in Legume Science" (Ed. R. Summerfield and A.H. Bunting, Royal Botanic Gardens, Kew U.K. pp. 597-601 (1980)

32. NifTAL Project, "Planning an international network of legume inoculation trials", University of Hawaii, and USAID (1979)

33. M.H. Ahmad, A.R.J. Eaglesham, S. Hassouna, B. Seaman, K. Ayanaba and E.L. Pulver, Trop. Agric. (Trinidad) 58:325-335 (1981)

34. R.A. Date, J. Aust. Inst. Agric. Sci., 35:27-37 (1969)

35. Indian Standards Institution, specification for Rhizobium inocula IS:8268-1976 (1976)

36. W.T. Scudder, Proc. Farmer. 93:72-73 (1978).

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The data presented here confirm those of others that N benefit may accrue to a cereal by intercropping with a grain legume. This phenomenon is significant to agricultural productivity only where levels of available N are low—a condition that often affects farmers in the tropics.

REFERENCES

Bartholomew, W.V. (1977) Soil nitrogen changes in farming systems in the humid tropics. *In*: Biological nitrogen fixation in farming systems of the tropics, A. Ayanaba & P.J. Dart (Eds.). Wiley, New York, NY, USA. Pp. 29-41.

Eaglesham, A.R.J., Minchin, F.R., Summerfield, R.J., Dart, P.J., Huxley, P.A., & Day, J.M. (1977) *Expl. Agric.* 13, 369-380.

Finlay, R.C. (1975) *INTSOY Series* 6, 77-85.

Ham, G.E. (1977) The acetylene-ethylene assay and other measures of nitrogen fixation in field experiments. *In*: Biological nitrogen fixation in farming systems of the tropics, A. Ayanaba & P.J. Dart (Eds.). Wiley, New York, NY, USA. Pp. 325-334.

Hardy, R. W. F. & Havelka, U.D. (1976) Photosynthate as a major factor limiting nitrogen fixation by field-grown legumes with emphasis on soybeans. *In*: Symbiotic nitrogen fixation in plants, P.S. Nutman (Ed.). Cambridge University Press, Cambridge, England. Pp. 421-439

Papers presented at a Workshop, 9-13 mar 1981, Cali, Colombia (Graham, P.H., and Harris, S.C., eds.)