Collecting *Rhizobium*, *Frankia* and mycorrhizal fungi

26

R.A. Date
Division of Tropical Crops and Pastures, CSIRO, Cunningham Laboratory, 306 Carmody Road, St Lucia, Queensland, 4067 Australia.

Introduction

Symbioses between higher plants and bacteria or fungi are known to be often important, and perhaps essential in some cases, for good plant growth. This is generally recognized to be due to improved nutrition of the host plant. Satisfactory exploitation of new plant germplasm in new environments, therefore, may depend on the natural presence of a suitable microsymbiont or its simultaneous introduction. Experience in recent years suggests that there is a close genetic association between host plant and microsymbiont (Date *et al.*, 1979). Thus, when germplasm of some plant species is being collected for use in new environments, it is recommended that symbiotic microorganisms also be collected.

The most important microsymbionts include:

- the root-nodule bacteria, *Rhizobium* and *Bradyrhizobium*, for nitrogen fixation in legumes (Dobereiner and Campelo, 1977; Mulder *et al.*, 1977; Peoples and Herridge, 1990);
- vesicular–arbuscular mycorrhiza (VAM) for phosphorus supply in many plants (Hayman, 1982);
- actinorhizal associations (*Frankia*) for nitrogen supply in about 200 species, including forages and forestry species such as *Alnus*, *Allocasuarina*, *Elaeagnus*, *Hippophae*, *Purshia* and *Shepherdia* (Becking, 1977; Akkermans and van Dijk, 1981);
- ectomycorrhiza (over 1000 species of basidiomycetes and ascomycetes, mainly the former) for water and nutrient uptake in many forestry species in the families Pinaceae, Betulaceae, Salicaceae, Myrtaceae, Casuarinaceae and some Caesalpiniaceae and Dipterocarpaceae (Molina and Trappe, 1982).

Many of the early studies of inoculation with a microsymbiont

involved the transfer of soil from established crops, plantations and forests. Today, however, most microsymbionts can either be cultivated on artificial media or maintained as 'enriched' cultures in association with an appropriate host nurse-plant in controlled conditions. These provide a good source of inoculum that can be introduced into the soil with the seeds at sowing, applied either separately or directly to the seed.

Some of the practical problems of collecting symbiotic microorganisms are outlined in this chapter as a guide to the plant germplasm collector undertaking this work for the first time. The approach and methodologies described define principles and can be altered to suit individual requirements and facilities.

Pre-sampling considerations

Suitable isolates of the target microsymbiont may already be available. To ascertain whether this is the case, the first step is to examine the catalogues of existing bacterial and fungal collections. Some idea of the holdings of collections may be obtained from the *World Directory of Collections of Cultures of Microorganisms* (Staines *et al.*, 1986). This information is also available both on-line and on disk. Bower (1989) reviews sources of information on microorganisms (see also Box 26.1). A Compact Disk Read-Only Memory (CD-ROM) called CD-STRAINS holds data on the holdings of the American Type Culture Collection, the Japanese Collection of Microorganisms and the Institute of Fermentation, Osaka. For information on root-nodule bacteria of legumes, perhaps a better starting-point is McGowan and Skerman (1986). Carlowitz (1991) lists suppliers of microsymbiont inoculants for multipurpose trees and shrubs. Hall and Minter's (1994) *International Mycological*

Box 26.1
Information sources on microbial collections

The World Data Centre of the World Federation of Culture Collections, located in Japan, has information on over 320 culture collections, forming the basis of Staines *et al.* (1986). It is a cosponsor of the Microbial Strain Data Network (MSDN).

Many collections worldwide, including informal research collections, are linked through MSDN. Its core service is access to databases, electronic bulletin boards and directories, via Dialcom.

The United Nations Environment Program (UNEP) and the United Nations Educational, Scientific and Cultural Organization (Unesco) have supported the development of a 20-country network of 24 microbiological resource centres (MIRCEN). These preserve, identify and distribute microbial germplasm, as well as providing training and resources in some cases. The MIRCEN in Stockholm has lately taken on an informatics role.

Directory brings together information on over 280 mycological organizations and institutions worldwide.

If potentially useful strains are not already available, one must decide where to collect them. Prospecting for microsymbionts should concentrate on the same geographic and ecological areas from which the plant germplasm to be inoculated was collected. Edaphic factors are particularly important in identifying areas where suitable strains would be most likely to be found (Date *et al.*, 1979; Molina and Trappe, 1982).

A perhaps more common situation, however, is for plant germplasm and microsymbiont collecting to occur in tandem. For example, a plant germplasm collector may also gather nodule samples and return them to a colleague in a microbiology department or institute for isolation of the microorganism. Joint collecting trips may be required when it is necessary to isolate from fresh nodule or root material.

Ideally, the microsymbiont and seeds should be collected from the same plant. However, this is often difficult, since seed collecting is often best done at times when the host plant has a reduced complement of active nodules or mycorrhiza. For example, nodules from annual legumes are best obtained from seedlings, though the nodule population in young plants may be composed of a different suite of strains from that of older plants as seasonal and soil conditions change (Caldwell and Weber, 1970; Weber and Miller, 1972; R.A. Date, unpublished data for strains of *Bradyrhizobium* forming nodules on *Stylosanthes*), whereas seeds are not available until plant maturity. In long-lived perennial plants, maximum seed set is often in the dry season, when nodules and fungi are not easily found. The presence of nodules on the root system in nitrogen-fixing symbioses is often seasonally dependent, especially if soil nitrogen is at levels adequate for good plant growth. For example, *Leucaena* growing as a closed-canopy stand was observed to have nodules at 98 days, but not at 205 or 274 days, with nodules reappearing on the root system at 423 days (Wong *et al.*, 1989). Sampling of soil and roots for ectomycorrhizal organisms is best done in the spring, when plants are actively growing. Young sporocarps are preferred for isolation but it is important to collect fully mature material for reliable identification of the species. Ectomycorrhizal numbers and sporocarp production also vary seasonally (Grand and Harvey, 1982).

Arrangements for the isolation and storage of the microsymbiont material should be made before setting out. When collecting abroad, it is essential to clear and coordinate microsymbiont collecting activities with the relevant local authorities, in the same way as is done for plant germplasm (Chapters 2 and 3). Microsymbionts are just as much part of biodiversity as the plants on which they live. Material collected by a national programme in its own country may need to be isolated or stored abroad, if adequate facilities are not available locally, and the isolates reimported as and when necessary. Quarantine clearance for the import and export of microsymbiont material may be necessary

and the question should be investigated before setting out to collect (Chapter 17).

Types of sample

Microsymbionts can be isolated from nodules, roots or soil. For the root-nodule bacteria of legumes and for many actinorhizal organisms, the best source is obviously nodules obtained directly from the plant from which seeds are also being collected. When nodules are not available, collectors should sample a small piece of root and/or soil from near the root. These can be used to inoculate seedlings of the host plant growing aseptically to obtain fresh nodules, from which isolates can be made. However, this is a method of last resort, because it will not necessarily yield nodules of those strains that would have formed in the field. This may be significant, depending on the exact purpose for which nodules are being collected. If, for example, specific collecting is being carried out for a strain with the ability to nodulate a host at low soil pH or for a strain that is highly competitive for nodule formation in the presence of large populations of ineffective strains, then reproducing these conditions for aseptically growing seedlings may not be possible.

Collecting soil and/or root pieces is the only way of obtaining material of those microsymbionts which do not form nodules or that cannot be grown easily in axenic culture, e.g. *Glomus* (VAM) and some ectomycorrhizal organisms. In these cases, pure or enriched cultures may be obtained by collecting spores by a wet sieving method (e.g. Gerdemann and Nicholson, 1963; Hayman, 1982; Molina and Palmer, 1982; Beaton *et al.*, 1985).

Sampling guidelines

Plants typical of the area should be selected for nodule sampling. Collecting along roadsides or disturbed areas must be avoided, unless, of course, such habitats are typical of the species. Ideally, each sample of microsymbiont should be unique and originate from a single plant. It should also be associated with a single-plant seed sample, as symbiotic microorganism and host are often closely affiliated genetically (Date *et al.*, 1979). Recent experience indicates that 10–20 legume nodules per plant should be collected to adequately sample the variation in strain types in the nodule population. It is a good idea to collect two to five such samples to represent a single site, as not all nodules will yield a viable culture and only a small proportion (as low as 5%) of the nodules may contain an effective nitrogen-fixing strain. Similar guidelines apply to collecting *Frankia*, VAM and ectomycorrhizal fungi. Usually, it is only necessary to process about half the sample, keeping the other half

as reserve against accidental loss during surface sterilization and isolation.

It is important in collecting microsymbionts to know where to look on or about the root system for nodules, rhizomorphs and sporocarps. The location of nodules on the root system is very species-specific and is further influenced by local conditions. In small, strongly tap-rooted species like *Trifolium semipilosum*, nodules can be found within 4–5 cm of the root crown. In *Stylosanthes*, nodules are more or less equally distributed along the length of the root system to a depth of 15 cm, but may be deeper in species like *S. capitata* growing in acid sandy soils (Venezuela) or oxisols (Brazil). *Acacia* and *Casuarina* species growing in free-draining deep sands have nodules at depths of 1–2 m, which is probably related to the level of the water-table.

Small herbaceous legumes (e.g. *Trifolium semipilosum*, *Lotus* spp.) can be sampled by digging a small core (diameter 10–15 cm, depth 10 cm) around the tap root. For shrubs and trees, it is rarely necessary to excavate the entire root system. Careful removal of soil from around the root crown with a knife or small trowel usually provides good results. Most nodules are located on adventitious roots in the top 5 cm of soil. For seedlings, a small core (as for herbaceous legumes) of soil around the root is usually adequate, but on mature plants nodules may be found further out and much deeper, depending on conditions. Usually, it is possible to collect nodules from the cores by sequentially fragmenting the soil by hand, but it may be necessary for dry or clay soils to soak the core of soil in water and allow the soil to fall away. Alternatively, and especially in species where nodules become detached readily from fine adventitious roots (e.g. *Macroptilium*, *Leucaena*), soil and roots with nodules can be washed over a sieve (1–2 mm openings). In either case, nodules can be recovered with forceps. When the sample is to be stored in vials with desiccant, it is advisable to dry the nodules with a paper towel before placing in the vial. (For equipment needed, see Box 26.2.)

Nodule size and shape are also very species-specific. Those of *Arachis* and *Stylosanthes* are 1–2 mm in diameter and firmly attached, whereas those of *Alnus*, *Casuarina*, *Macroptilium* and *Medicago* are typically larger and become detached very readily. The shape of legume nodules is governed by the extent and location of the meristem. Hemispherical peripheral meristems produce spherical nodules, as typified by *Arachis*, *Glycine*, *Lotus*, *Macroptilium*, *Stylosanthes* and *Vigna*. Elongated cylindrical forms result from growth of apical meristems, which may divide, giving digitate or even coralloid nodules, as observed in *Leucaena*, *Trifolium* and *Vicia*. When meristems divide laterally, subsequent growth of the nodule tends to surround the roots, as in *Lupinus* (Corby, 1971; Allen and Allen, 1981). Nodules with peripheral meristems are sometimes referred to as determinate and the other types as indeterminate. In cross-section, determinate nodules show a single circular active bacteroid (red) zone, whereas in indeterminate nodules there are one or more irregularly shaped active bacteroid

Box 26.2
Equipment required for collecting microsymbionts

- Digging implement (trowel, strong knife).
- Secateurs or strong scissors.
- Forceps, scalpel or small sharp knife.
- Hand lens.
- Collecting vials with desiccant.
- Waxed paper bags.
- Paper towels.
- 5 l plastic container.
- Permanent marker pen.
- Adhesive tape.
- Plastic bags.
- Containers for storage of samples.
- Cooler box (optional).
- See Date and Halliday (1987) for details of portable kits if isolation of microsymbiont is to be attempted in the field.

zones. Nodules on most non-leguminous plants are modified lateral roots with slow-growing meristems, usually branching dichotomously to give coralloid-type nodules up to 5–6 cm long, as typified by *Alnus*, *Casuarina* and *Ceanothus* (Becking, 1977).

Endomycorrhizal plants have no obvious macroscopic structures, but with experience hyphal growth on the roots may be recognized with a hand lens. There is wide variation in the morphology of ectomycorrhiza. They appear as a continuation of the root in *Pinus taeda*, but coralloid forms on *P. strobus* and *P. resinosa* are more like the root-nodules of *Lupinus* (Grand and Harvey, 1982). In other host species the mycorrhiza varies from a thin mantle of fungal hyphae to thickened zones of terminal roots (Chilvers and Pryor, 1965). In *Acacia* and *Nothofagus* species gasterocarps up to 3 cm in diameter may be found in the surface 10 cm of soil, usually on the side exposed to the prevailing winds, within 1.5 m of the trunk. They are usually most plentiful in late autumn, winter and early spring (Beaton *et al.*, 1985).

Storage guidelines

The viability of *Rhizobium*, *Bradyrhizobium* and *Frankia* in the nodule sample is variable and it is best to isolate on the day of collecting or soon thereafter. When this is not possible, nodules must be dried. The microsymbiont will usually remain viable in the dried state for several weeks, with 75–90% of nodules yielding viable isolates after reimbibition and surface sterilization.

Drying is best achieved by placing nodules in an airtight vial or screw-capped bottle with a desiccant such as anhydrous $CaCl_2$ or silica gel (Date and Halliday, 1987). Washing samples should be avoided, but, if it is necessary to wash, then nodules should be dried with absorbent paper before being placed in the vial. The desiccant can be kept in place by a plug of cotton or absorbent paper. Its volume should exceed that of the sample. A 10–20 ml capacity vial with 25% of its volume filled with desiccant and 10% made up of a cotton or paper plug is a good container for preserving and transporting samples. Vials should be kept cool.

There are some species, however, where the proportion of recovery is less than 5% of nodules and it is therefore necessary to isolate from fresh nodules. For example, no viable isolates of *Bradyrhizobium* were obtained from more than 150 dried nodule samples of *Stylosanthes capitata* collected in Brazil and processed in Australia six to ten weeks later, but isolation was 65–80% successful for fresh nodules processed the day of collecting (R.A. Date, unpublished data). When isolations are to be made on the day of collecting, the simplest method is to gather the whole or part of the root system into sealable polythene bags. On return to a laboratory, roots and nodules must be washed free of soil and the nodules excised with a short piece of root attached. This is important, especially for small nodules, as it reduces the entry of sterilant during surface sterilization. Portable isolating kits are described by Date and Halliday (1987).

Unlike nitrogen-fixing root-nodules, sporocarps should be kept dry, but not airtight. Waxed paper containers are usually used (Molina and Palmer, 1982).

Data collecting

Because of the close association between environmental, especially soil, features and various traits and properties of the microsymbiont (Date *et al.*, 1979; Molina and Trappe, 1982), it is important to record some descriptive information on the site. Experience suggests that the kinds of site data normally recorded by plant germplasm collectors are also relevant to collecting microsymbiont germplasm. These have been described in several publications (Mott and Jimenez, 1979; Date and Halliday, 1987; Stowers, 1987) and are also considered in detail in Chapter 19.

Isolation of microsymbionts

Successful isolation of microsymbionts depends on the quality of the sample. Damaged *Rhizobium*, *Bradyrhizobium* or *Frankia* nodules are not satisfactory and should be used only to inoculate aseptically growing

seedlings of the host plant to obtain fresh nodules. Dried nodules need to be reimbibed for 30–60 minutes before surface sterilization. Methods of sterilization and isolation vary with individual requirements and with the microsymbiont. A number of laboratory procedures have been described for the following organisms:

- *Rhizobium* and *Bradyrhizobium*: Brockwell (1980), FAO (1983), Somasegaran and Hoben (1985), Date and Halliday (1987);
- VAM: Daniels and Skipper (1982), Hayman (1982);
- *Frankia*: Stowers (1987);
- ectomycorrhiza: Molina and Palmer (1982).

It is essential to confirm that isolates are representative, pure cultures and able to form nodules (or infect a host root system) on aseptically growing host plants and thus satisfy Koch's postulates regarding causal organisms. Isolates must also be adequately preserved, evaluated and documented. Methods and criteria are described by Vincent (1970), Brockwell (1980), FAO (1983) and Date and Halliday (1987).

Acknowledgements

I am indebted to Dr P. Reddell for advice on collecting mycorrhizal fungi.

References

Akkermans, A.D.L. and C. van Dijk (1981) Non-leguminous root nodule symbioses with actinomycetes and *Rhizobium*. In: Broughton, W.J. (ed.) *Nitrogen Fixation*. Vol. 1. pp. 57–103. Clarendon Press, Oxford.

Allen, O.N. and E.K. Allen (1981). *The Leguminosae. A Source Book of Characteristics, Uses and Nodulation*. University of Wisconsin Press, Wisconsin.

Beaton, G., D.N. Pegler and T.W.K. Young (1985) Gasteroid basidiomycota of Victoria State, Australia: 8–9. *Kew Bulletin* 40:827–842.

Becking, J.H. (1977) Dinitrogen fixing associations in higher plants other than legumes. In: Hardy, R.W.F. and W. Silver (eds) *A Treatise on Dinitrogen Fixation*. pp. 185–275. John Wiley & Sons, New York.

Bower, D.J. (1989) Genetic resources worldwide. *Tibtech* 7:111–116.

Brockwell, J. (1980) Experiments with crop and pasture legumes - principles and practice. In: Bergersen, F.J. (ed.) *Methods for Evaluating Biological Nitrogen Fixation*. pp. 417–490. John Wiley & Sons, New York.

Caldwell, B.E. and D.F. Weber (1970) Distribution of *Rhizobium japonicum* serogroups in soybean nodules as affected by planting dates. *Agronomy Journal* 62:12–14.

Carlowitz, P.G. von (1991) *Multipurpose Trees and Shrubs - Sources of Seeds and Inoculants*. ICRAF, Nairobi.

Chilvers, G.A. and L.D. Pryor (1965) The structure of eucalypt mycorrhizas. *Australian Journal of Botany* 13:245–259.

Corby, H.D.L. (1971) The shape of leguminous nodules and the colour of leguminous roots. *Plant and Soil* (Special Volume) 305–314.

Daniels, B.A. and H.D. Skipper (1982) Methods for the recovery and quantitative estimation of propagules from soil. In: Schenck, N.C. (ed.) *Methods and Principles of Mycorrhizal Research*. pp. 29–35. American Phytopathological Society, Minnesota.

Date, R.A. and J. Halliday (1987) Collecting, isolation, cultivation and maintenance of rhizobia. In: Elkan, G.H. (ed.) *Symbiotic Nitrogen Fixation Technology*. pp. 1–27. Marcel Dekker, New York.

Date, R.A., R.L. Burt and W.T. Williams (1979) Affinities between various *Stylosanthes* species as shown by rhizobial, soil pH and geographic relationships. *Agro-Ecosystems* 5:57–67.

Dobereiner, J. and A.B. Campelo (1977) Importance of legumes and their contribution to tropical agriculture. In: Hardy, R.W.F. and A.H. Gibson (eds) *A Treatise on Dinitrogen Fixation*. pp. 191–220. John Wiley & Sons, New York.

FAO (1983) *Technical Handbook on Symbiotic Nitrogen Fixation*. FAO, Rome.

Gerdemann, J.W. and T.H. Nicholson (1963) Spores of mycorrhizal *Endogene* extracted from soil by wet sieving and decanting. *Transactions of British Mycological Society* 46:235–244.

Grand, L.F. and A.E. Harvey (1982) Quantitative measurement of ectomycorrhizae on plant roots. In: Schenck, N.C. (ed.) *Methods and Principles of Mycorrhizal Research*. pp. 157–164. American Phytopathological Society, Minnesota.

Hall, G.S. and D.W. Minter (1994) *International Mycological Directory* 3rd edition. CAB International, Wallingford.

Hayman, D.S. (1982) Practical aspects of vesicular-arbuscular mycorrhiza. In: Subba Rao, N.S. (ed.) *Advances in Agricultural Microbiology*. pp. 325–373. Butterworths, London.

McGowan, V.F. and V.B.D Skerman (1986) *World Catalogue of* Rhizobium *Collections*. World Data Center, University of Queensland, Brisbane.

Molina, R. and J.G. Palmer (1982) Isolation, maintenance and pure culture manipulation of ectomycorrhizal fungi. In: Schenck, N.C. (ed.) *Methods and Principles of Mycorrhizal Research*. pp. 115–129. American Phytopathological Society, Minnesota.

Molina, R. and J.M. Trappe (1982) Applied aspects of ectomycorrhizae. In: Subba Rao, N.S. (ed.) *Advances in Agricultural Microbiology*. pp. 305–424. Butterworths, London.

Mott, G.O. and A. Jimenez (1979) *Handbook for the Collection, Preservation and Characterization of Tropical Forage Germplasm Resources*. CIAT, Cali.

Mulder, E.G., T.A. Lie and A. Houwers (1977) The importance of legumes under temperate conditions. In: Hardy, R.W.F. and A.H. Gibson (eds) *A Treatise on Dinitrogen Fixation*. pp. 221–242. John Wiley & Sons, New York.

Peoples, M.B. and D.H. Herridge (1990) Nitrogen fixation by legumes in tropical and subtropical agriculture. *Advances in Agronomy* 44:155–223.

Somasegaran, P. and H.J. Hoben (1985) *Methods in Legume*–Rhizobium *Technology*. NifTAL, University of Hawaii, Honolulu.

Staines, J.E., V.F. McGowan and V.B.D. Skerman (1986) *World Directory of Collections of Cultures of Microorganisms*. World Data Center, University of Queensland, Brisbane.

Stowers, M.D. (1987) Collection, isolation, cultivation and maintenance of *Frankia*. In: Elkan, G.H. (ed.) *Symbiotic Nitrogen Fixation Technology*. pp. 29–53. Marcel Dekker, New York.

Vincent, J.M. (1970) *A Manual for the Practical Study of Root-Nodule Bacteria*. Blackwell Scientific Publications, Oxford.

Weber, D.F. and V.L. Miller (1972) Effect of soil temperature on *Rhizobium japonicum* serogroup distribution in soybeans. *Agronomy Journal* 64:796–798.

Wong, C.C., J. Sundram, R.A. Date and R.J. Roughley (1989) Nodulation of *Leucaena leucocephala* in acid soils of Peninsular Malaysia. *Tropical Grasslands* 23:171–178.

Useful addresses

MSDN Secretariat
307 Huntingdon Road
Cambridge CB3 0JX
UK
Tel: +44 1223 276622
Fax: +44 1223 277605
E-mail: msdn@bdt.ftpt.br

Unesco Microbial Resources Center
Karolinska Institute
S 104 10 Stockholm
Sweden
Tel: +46 8 7287147
Fax: +46 8 331547
E-mail: eng-leong__foo__ba@kicom.ki.se