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Specificity among the Casuarinaceae in root nodulation by *Frankia*

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Abstract

Pure cultured isolates of *Frankia* made from root nodules of plant species from among three genera of the host family Casuarinaceae were used in inoculation trials of seedlings grown in water culture. A large number of host species among the genera Allocasuarina, Casuarina and Gymnostoma from Australia, Papua New Guinea and other South Pacific Islands were tested. The most widely infective *Frankia* strains were CcI3 and AllI1; the *Frankia* strains with the narrowest host range within the Casuarinaceae were CcI2 and GpI1. Intrafamily cross-inoculations were uncommon. The most broadly receptive host species was *G. papuanum*. For many species of *Allocasuarina* tested, no infection by any *Frankia* available for testing could be observed.

Introduction

The woody dicotyledonous family Casuarinaceae includes about ninety species of trees and shrubs with a natural distribution in Australia and parts of Asia and Oceania. Four genera have been named by Johnson (cf. Torrey and Berg, 1988). Several species among these genera have been planted extensively in tropical and sub-tropical areas of the world for multiple uses, including fuelwood, windbreaks, soil improvment and soil or dune stabilization. Many species show the capacity to tolerate infertile, saline and arid sites, in part, because of symbiotic nitrogen fixation attributable to root nodulation by the actinomycete *Frankia*.

The first isolation and culture of an infective and effective strain of *Frankia* from root nodules in the family Casuarinaceae was reported by Diem *et al.* (1983a, b). Their strain Cj1–82, (catalog number ORS 021001) was isolated from root nodules of *Casuarina junghuhniana*. The following year Zhang *et al.* (1984) reported the isolation and culture of *Frankia* strains CcI2 (catalog number HFP020202) and CcI3 (catalog number HFP020203) from root nodules of *Casuarina cunninghamiana*. Since these early successes, nearly two dozen pure cultured isolates of *Frankia* have been made from root nodules of members of the family Casuarinaceae and their infectivity on host plants reported (see Table 1).

Inoculation with crushed nodule suspensions

Prior to 1983 inoculation of host plants of the genera of the family Casuarinaceae was achieved with the use of preparations of crushed or ground nodule suspensions. Seeds of Casuarina do not carry on their surfaces the *Frankia* that will successfully initiate nodule formation on the seedlings. Therefore, successful nodulation of members of this family depends upon deliberate transfer of inoculum with any seeds to be planted in locations where Casuarina did not previously exist (a common event in the early 1900's when members of the family were experimentally introduced into tropical and sub-tropical countries around the world from seed originating in Australia or the South Pacific Islands). Early in such experiments it was recognized that a specificity of host plant—soil microorganism interaction existed and that care was needed in the selection of inoculum if successful nodulation and therefore plant establishment was to be achieved. There are no records on the inocula-

tion procedures and source of inoculum in early plantations of *Casuarina equisetifolia*, *C. cunninghamiana*, and *C. glauca* around the world. It must be assumed that in the early experimental trials of members of the family for forestation, dried nodules or soil from around plants originating in Australia were carried with seeds on export.

One of the early studies of host specificity in the family Casuarinaceae was made by Mowry (1933, 1934) in South Florida. Earlier introductions of a number of species into U.S.D.A. plantations at Homestead, Florida provided him with nine named species in the family with established nodulated adult plants. His experiments on cross-inoculation within the group were conducted by collecting fresh nodules, surface-sterilizing the nodules and placing nodule pieces around roots of seedlings growing in sterile soil. After 2-1/2–6 months, nodules could be observed on the previously unnodulated plants. He found that root nodules from one species of *Casuarina* (probably *C. cristata* called by him *C. lepidopholia*) effectively nodulated seedlings of other species in this genus including *C. equisetifolia* *C. glauca*, *C. cunninghamiana* and several other species of uncertain identity. He concluded that within the genus Casuarina successful cross-inoculation occurred. Parker (1932) reported that nodule suspensions from one species of *Casuarina* served as an infective inoculum on all *Casuarina* species he tested (cited by Coyne 1973).

Further studies of cross-inoculation in the family were reported by Coyne (1973, 1983). He made field collections of root nodules of six species of *Casuarina* and *Allocasuarina* and performed cross-inoculation trials on seedlings of all six species. Three of the species, *C. glauca*, *C. cunninghamiana* and *C. cristata* belonged to the genus Casuarina, while three species fall in the group described by Johnson (1982) as Allocasuarina, *viz. A. torulosa*, *A. littoralis* and *A. verticillata* (formerly *C. stricta*). According to Coyne, the three species of Casuarina responded most rapidly to inoculation with crushed nodule suspensions, especially with inoculum prepared from Casuarina. Allocasuarina species were slower to nodulate whatever the inoculum and *A. littoralis* failed to form nodules. Overall, *C. glauca* crushed nodules served as the most infective inoculum. In general, best results involved using as inoculum nodule suspensions originating from the same host species. Some degree of host specificity was apparent in these trials.

In very carefully performed cross-inoculation trials Reddell and Bowen (1985a, b, 1986) used crushed nodule suspensions prepared from axenically grown seedlings previously inoculated with surface-sterilized nodules from five different sources. *C. equisetifolia* and *C. cunninghamiana* seedlings reponded differently to the different nodule suspensions indicating considerable speificity in infective capacity. Thus, for example, seedlings of *C. cunninghamiana* failed to nodulate when inoculated with nodule suspensions that effectively nodulated *C. equisetifolia*.

In field trials on Casuarina establishment throughout the tropical world the general rule of thumb has been to inoculate seedlings from root nodule suspensions or soil from around effectively nodulated plants of the same species, if possible.

There is difficulty in interpreting results of cross-inoculations based on crushed nodule suspensions as was noted earlier by VandenBosch and Torrey (1983). Even careful sterilization of the surfaces of nodules used for inoculum does not preclude the possibiity that more than one genetic strain of *Frankia* occurs within the nodule (Benson and Hanna, 1983; Reddell and Bowen, 1985b).

Inoculation with pure cultured Frankia isolates

With the availability of pure cultured isolates from root nodules of the family Casuarinaceae, we undertook to make as complete an analysis of the host-specificity of the host plant-*Frankia* interactions within the family as we could perform. We used standard conditions of water culture (VandenBosch and Torrey, 1983) to grow seedlings of eight species among three genera of the family including species of *Casuarina*, *Allocasuarina* and *Gymnostoma*. Six *Frankia* strains isolated, cultured and characterized in earlier studies were used in homogenized cell suspensions as inoculum. After an appropriate time to allow for nodule development, the cross-inoculation trials were scored for infectivity and nodulation. The results of these trials are summarized in Table 2.

In addition to the host species listed in Table 2 we made extensive attempts to nodulate plants from a

Table 1. Cross-inoculation studies involving pure cultured strains of *Frankia* isolated from root nodules of members of the Casuarinaceae. Host plants tested included seedlings from the three families Casuarinaceae, Elaeagnaceae and Myricaceae. Catalog numbers are listed by Lechevalier (1985). Scores: + = all plants nodulated, +/− = less than 50% nodulated, − = no nodulation, space = not tested

Trivial designation	Catalog number	Host of origin	*C. cunn.*	*C. equis.*	*C. glauca*	*Gym. pap.*	*E. ang.*	*E. umb.*	*Hip. rh.*	*Shep. ar.*	*M. gale*	*M. cerf.*	References
Allocasuarina lehmannia													
AllI1	HFP022801	+	+	+	+	+/−	−	−		−	+		Zhang and Torrey, 1985
Casuarina cunninghamiana													
CcI2	HFP020202	−	−	−	−	+	+	+		+	+		Zhang *et al.*, 1984
CcI3	HFP020203	+	+	+	+	+/−	−	−		−	+	+	Zhang *et al.*, 1984
R43	LLR02022	−	−	−			+	+	+			+	Lechevalier *et al.*, 1987
Casuarina equisetifolia													
CeI2	DDB020210	−		−			+					+	Baker, 1987
	DDB020510	−		−			+					+	Baker, 1987
CeI5	UFG		−		+/−	+	+	+/−			+	?	Berg, unpubl.
D1	ORS020601	−		−									Diem *et al.*, 1982
D11	ORS020602	−		−			+		+				Gauthier *et al.*, 1981
G2	ORS020604	−		−			+		+				Gauthier *et al.*, 1983
Ce D	ORS010606	+		+									
Ce F	ORS020607	+	+	+	+								
JCT287	——	+	+	+	+								Rosbrook and Bowen, 1987
CeC14	——	+/−		+/−									Huang *et al.*, 1985
Casuarina glauca													
CAQP1	——	+			+								Chaudhary and Mirza, 1987
CAQP2	——	+			+								Chaudhary and Mirza, 1987
CAQP3	——	−			−								Chaudhary and Mirza, 1987
CAQP4	——	−			−								Chaudhary and Mirza, 1987
CAQP5	——	−			−								Chaudhary and Mirza, 1987
CgI1	UFG	+/−	−		+/−	+	+				+	?	Berg, unpubl.
Casuarina junghuhniana													
Cjl-82	ORS021001	+	+	+	+								Diem *et al.*, 1983a
JCT295	--		+		+								Rosbrook and Bowen, 1987
Gymnostoma papuanum													
GpI1	HFP021801	+	−	−	−	+	+	+		+	+		Racette and Torrey, 1989

Table 2. Host specificity in the Casuarinaceae as demonstrated by nodulation response of seedlings of species from three genera of the family. Inoculation was with suspensions of pure cultured strains of Frankia isolated from root nodules within the family

Host species	*Frankia* inoculum					
	CcI2	CcI3	AllI1	CeI5	CgI1	GpI1
Casuarina cunninghamiana	−	+	+	−	−	−
Casuarina equisetifolia		+	+			
Casuarina glauca		+	+	+/−	+/−	
Allocasuarina verticillata	−	+/−	+/−	−	−	−
Allocasuarina decaisneana		+/−	+/−	−	−	−
Gymnostoma papuanum	+	+/−	+/−	+	+	+
Allocasuarina torulosa	−	+/−	+/−			−
Allocasuarina paludosa	−	+/−	+/−			−
Allocasuarina lehmanniana		−	+			
Allocasuarina fraserana	−	−	−			−
Allocasuarina spp.: (ten additional species tested, all negative)						

+ = plant nodulated, +/− = less than 50% nodulated, − = no nodulation. space = not tested.

list of species of *Allocasuarina* by inoculation with available isolates. *Frankia* strains CcI2, CcI3, AllI1 and GpI1 all proved to be non-infective in the following host species: *A. acutivalvis*, *A. corniculata*, *A. distyla*, *A. fraserana*, *A. humilis*, *A. littoralis*, *A. moniflora*, *A. nana*, *A. pinaster*, *A. rigida* and *A. scleroclada*.

A number of interesting comparisons and observations can be made from the data displayed in Tables 1 and 2. Consider first the results shown in Table 2 in which the tests made were all within the family Casuarinaceae. Looking at host susceptibility to infection, *Gymnostoma papuanum* is unique within the group tested as it is nodulated by all of the six isolates from the three host genera. *Gymnostoma papuanum* is what has been termed by Baker (1987) a promiscuous host. No other species within the family has shown such susceptibility. On the other extreme were the many species of *Allocasuarina* that failed totally to respond to all the *Frankia* strains tested. Since many of these species have been reported to be nodulated in the field (cf. Torrey, 1983), it must be presumed that we have failed thus far to isolate a *Frankia* strain capable of infecting these host plants. *A. lehmanniana* is of interest in this connection as it is infected only by the *Frankia* strain AllI1 isolated from its nodules. This case represents the most specific host-microorganism association in Table 2.

It is apparent in Table 2 that *Frankia* strains CcI3 and AllI1 from different host genera have a fairly broad range of infective capacity within the family, nodulating all plants tested (+) or a fraction of plants tested (+/−) in species of the three genera studied. *Frankia* strain GpI1 has one of the narrowest host ranges within the family, infecting only its original host species. This narrowness is in fact deceptive as we shall see in considering the wider group of host plants listed in Table 1. The infection of *G. papuanum* by GpI1 might be more usefully considered in terms of the promiscuity of the host plant rather than the specificity of the *Frankia* strain.

A similar pattern of specificity is seen also in the *Frankia* strain CcI2 which, despite its isolation from nodules of *Casuarina cunninghamiana*, fails to nodulate its host of origin or other *Casuarina* species tested. This type of isolate is of common occurrence and presents an anomaly needing explanation.

When one turns to Table 1, *Frankia* isolates of the type represented by CcI2 are seen to have been reported by four independent laboratories. In addition to CcI2 may be listed R43, also isolated from root nodules *C. cunninghamiana*, and CeI2, D11 and G2 isolated from *C. equisetifolia*. All of these isolates from *Casuarina* species have the following characteristics in common: they do not infect the host plants from which they were isolated, yet they effectively nodulate one to several different species in the unrelated family Elaeagnaceae, for example, Elaeagnus, Hippophaë or Shepherdia. Each of them is a pigmented isolate, usually pink, red or orange depending on the medium in which they are

cultured. These strains have other characteristics unlikely to affect their capacity to infect, such as the capacity to form vesicles in the presence of combined or reduced nitrogen compounds.

It should be noted that the infection process in all Casuarinaceae studied carefully shows that root hair infection is involved (Callaham *et al.* 1979). In contrast, infection in members of the Elaeagnaceae involve direct epidermal entry, a contrasting mode of infection (Miller and Baker, 1985, 1986).

At the present time no good explanation can be given for the occurrence of this type of isolate. One possibility is that root nodules contained more than one *Frankia* strain in the nodules from which they were isolated. A second possibility is that the *Frankia* isolated was induced to undergo mutation during isolation which changed its character including its infectivity. New experimentation will be needed to understand this phenomenon. There is probably no connection between the pigmented character and the infective capacity. Chaudhary and Mirza (1987) have described isolates of *C. glauca* root nodules that are pigmented or unpigmented that nodulate the host of origin or fail to do so whether or not they are pigmented (Table 1). The capacity of these strains to nodulate members of the Elaeagnaceae was not reported.

Another matter of considerable interest with respect to *Frankia*-host specificity is demonstrated in Table 1. It has been reported (Zhang and Torrey, 1985a) that CcI3, infective on its host of origin and on other *Casuarina* and *Allocasuarina* species, is also able to infect seedlings of *Myrica gale*. Myrica is a member of the family Myricaceae, unrelated in any clear way to the Casuarinaceae. Nodules of Casuarina and Allocasuarina show no vesicles in the infected cells and N_2 fixation proceeds in the presence of only filamentous cells of *Frankia* (Zhang and Torrey, 1985a, b). Root nodules of *M. gale* infected by CcI3 or AllI1 show club-shaped vesicles more or less characteristic of Myrica root nodules, a host-specific expression, and effectively fix dinitrogen (unpublished observations).

Berg (personal communication) has observed that his Casuarina isolate(s) from *C. equisetifolia* collected in S. Florida also effectively nodulate *Myrica cerifera*, a more southerly species of *Myrica*. His idea is that the native *M. cerifera* was nodulated long before the exotic Casuarina species were brought to Florida and that *Casuarina* species accepted the *Frankia* derived from *Myrica cerifera*. These speculations are of course not explanations of this striking anomaly among cross-inoculation capacities. Explanations at the level of cell-to-cell recognition and interaction are needed.

Discussion

In his study of cross-inoculation groups among *Frankia* strains, Baker (1987) included *Casuarina equisetifolia* in his host groups for trials and twelve strains of *Frankia* isolated from Casuarina. Only four of the Casuarina isolates nodulated *Casuarina equisetifolia*; all of the others noduled *Elaeagnus angustifolia* except two that were not tested. The present studies which focus on the family Casuarinaceae extend his studies considerably and raise a number of further problems and questions.

The first conclusion that confirms earlier views by Lechevalier and Lechevalier (1986), Baker (1987) and others is that infectivity of host plants does not offer a useful character for the systematic classification of *Frankia*. Even with our incomplete knowledge of host specificity that conclusion is clear.

A second conclusion worth stating is that it is too early in our investigations of host specificity of *Frankia* to make reliable broad generalizations. With the isolation of GpI1 from Gymnostoma we have a *Frankia* strain that nodulates a member of the Casuarinaceae, *i.e.*, *G. papuanum*, fails to nodulate Casuarina, also in the Casuarinaceae, but nodulates *M. gale* of the Myricaceae. Our Table 2 also presents cases of isolates that are remarkably restrictive in the hosts they will nodulate within the family. Thus, for example, GpI1, CcI2, CeI5 and CgI1 are good examples. Looked at from the host's point of view, some hosts are very precise in their specification of an infective *Frankia* strain as, for example, *Allocasuarina lehmanniana*. For other species of Allocasuarina we have yet to isolate an infective *Frankia* organism, — a situation that applies as well to actinorhizal host genera such as Ceanothus, Purshia and others.

What can we say about host specificity in the family Casuarinaceae? Unlike the family Elaeagnaceae in which cross-inoculation among genera and species is nearly complete (Huang *et al.*, 1985, provide exceptions), in the family Casuarinaceae

complete intrafamily compatibility does not exist. The genus Allocasuarina seems to be most specific within the family, showing the narrowest tolerance to infection, Casuarina is intermediate and Gymnostoma offers the broadest tolerance, accepting *Frankia* strains quite outside family origins. Thus, a generally usable *Frankia* strain for inoculation of all members of the family Casuarinaceae is not likely to be found.

The promiscuous host genus Gymnostoma originates in the humid tropics. The very restrictive *Allocasuarina* species in many cases occur in arid sites or are endemics from very limited and specific geographic habitats. It is conceivable that only very specific *Frankia* strains will be functional on these plants. Reports by Lawrie (1983) on the often sparse natural occurrence of nodulated species within the family would seem to be in agreement with these restrictions of the microsymbiont.

Table 2 poses another problem. Frequently in water-culture trials inoculation with a *Frankia* strain leads to an incomplete response scored here as +/−, representing usually less than 50% nodulation of the test plants. A similar problem is apparent in the data of Huang *et al.* (1985) and is commented on in the trials by Baker (1987). One could attribute this variability in host susceptibility to either physiological or genetic causes or even failure of the test system. Zhang and Torrey (1985b) reported that seedlings of *Allocasuarina lehmanniana*, either in the greenhouse or in growth chambers failed to nodulate following inoculation with *Frankia* strain AllI1 when grown in water culture. All plants nodulated effectively when grown in washed river sand with the same nutrient solution supplied. The basis of this difference in infection was not determined. Also it is not known whether the way in which a culture of *Frankia* is grown affects the infectivity of the culture. These questions need to be studied in a systematic way so that we can understand the reliability of our test procedures.

Another interesting question illustrated in Table 1 is why *Myrica gale* and other *Myrica* species are nodulated by Casuarina isolates. The simple answer is that Myrica falls into the class of promiscuous hosts. In our studies of cross-inoculation of Myrica seedlings by *Frankia* isolates we have tested ten strains of *Frankia* isolated and cultured from root nodules of nine different plant species from seven plant genera including Alnus, Comptonia, Myrica, Elaeagnus, Casuarina, Allocasuarina, and Gymnostoma and found 100% nodulation of *Myrica gale*. Because of that promiscuity, Myrica and Gymnostoma do not represent useful genera for selective cross-inoculation trials. We still, however, are faced with explaining the basis for this broad susceptibility.

Another puzzle seen in Table 1 that raises interesting speculations is the observation that isolations from root nodules of Casuarina frequently result in pure cultures of *Frankia* strains that do not nodulate the host of origin but effectively nodulate members of the Elaeagnaceae. Can one in the course of the isolation process modify the infective capacity of *Frankia* isolates by manipulation? Here again, further experimentation is needed.

On what does host specificity depend? Probably in most cases, the host plant specifies an acceptable micro-symbiont. For the promiscuous host, if the microorganism belongs to the actinomycetous genus *Frankia*, it may be enough to quality for entry — *i.e.*, to elicit root hair deformation, root wall penetration, and filamentous growth into the root cortex. To date we know of no promiscuous hosts which are infected by direct entry. All are by root hair infection, which is surprising since the latter mode seems more complicated. For fastidious hosts, Frankia must meet and overcome additional host barriers before it infects the root system. The nature of these barriers remains to be deciphered.

To what extent does host specificity depend upon a symbiotic relationship, *i.e.*, a mutual response in which both partners contribute to the successful infection? It seems reasonable that each infection must represent an initial recognition of some kind. In Rhizobium-legume associations these early recognition steps involve chemical signals with the host root releasing specific chemical products that trigger a response in the microbial partner (cf. Halverson and Stacey, 1986; Torrey, 1988). Thus far no evidence exists for a similar signal system involving Frankia but a search for such chemical exchanges seems appropriate. In actinorhizal plants it would seem reasonable that a chemical released by the host root may be the first event in signalling a soil-borne *Frankia*. In promiscuous hosts the signal must be broadly perceived by *Frankia* strains in the

soil and the host makes no further specification for infection. In narrowly restrictive relationships, either the *Frankia* or the host plant may present the next restrictive step. For example, the chemical signal from the host plant fails to activate a *Frankia* response in most strains.

In a susceptible strain, growth toward the host root is stimulated. The microorganism then elicits root hair deformation (Berry and Torrey, 1983; Berry *et al.*, 1986), making the host cell vulnerable to direct surface association with the *Frankia* which is followed by root hair cell-wall dissolution, leading to entry. Here specific cell-wall hydrolyzing enzymes may come into action that will define further the specificity in the infection. Growth of the actinorhizal filaments through the root hair, into the root cortex eliciting host cellular proliferation are further steps that may succeed or fail, completing the sequence of specific events essential for infection. Host specificity of this complex sequence of infection events may be expressed by positive or negative responses at each step so that one finds a whole spectrum of restrictions that defines the specificities that we see and study. To unravel these sequential events in each case will be necessary if we are to understand fully the basis of host specificity in the *Frankia*-actinorhizal association.

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