Leaf curl syndrome of pigeonpea (*Cajanus cajan* (L.) Millsp.) is a systemic response to effective nodulation by the *Rhizobium* strain IC3342

NARAYANA M. UPADHYAYA†§¶, J. V. D. KUMAR RAO‡, PETER J. DART†‖ and DAVID S. LETHAM†

† *Plant Molecular Biology/Plant Cell Biology Group, Research School of Biological Sciences, The Australian National University, A.C.T. 2601, Australia*; ‡ *International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), P.O. Patancheru, Hyderbad, India*

(*Accepted for publication April 1991*)

Rhizobium strain IC3342 is an unusual bacterium that causes a leaf curl syndrome in pigeonpea (*Cajanus cajan* Millsp.) Growth characteristics, plasmid profile, conserved *nif* and *nod* gene sequences, and nodulation host range of this strain resemble that of the fast-growing *Rhizobium* strain ANU240 (NGR234). Leaf curl induction occurred only in hosts effectively nodulated by this strain. A plasmid-cured, non-nodulating derivative failed to induce leaf curl symptoms. The strain IC3342 competed poorly with fast- and slow-growing root-nodule bacteria, but the observed nodule occupancy of 10% was enough to produce leaf curl symptoms. Suppression of nodule development by added inorganic nitrogen also prevented symptom expression. Approach grafting of a healthy pigeonpea plant and a plant with leaf curl symptoms resulted in the development of leaf curl symptoms on the growing shoots of the healthy plant within 8 days of graft union. Further symptom expression ceased after graft separation. Feeding xylem sap from the leaf curled plant to a healthy plant induced the initial symptom of the syndrome, bending of the growing leaf tip. We conclude that the leaf curl induction is a systemic response for which effective nodulation is an apparent prerequisite.

INTRODUCTION

Plant–bacteria interactions, particularly those involving *Rhizobium* and *Agrobacterium*, have been the subject of much recent research. Investigations of bacterially-induced plant cell growth and development have concentrated on the biological mechanisms modulating plant growth and on the genetic components involved in the process. The morphological and biochemical changes that occur during the infection process leading to the development of a functional, nitrogen-fixing nodule have been reviewed [*4*, *5*]. Whilst most *Rhizobium*–legume interactions are advantageous to plant growth under nitrogen-limiting conditions, in some cases, such as ineffective nodulation, there may

§To whom correspondence should be addressed.

Present addresses: ¶ CSIRO Division of Plant Industry, GPO Box 1600, Canberra ACT 2601, Australia; ‖ Department of Agriculture, University of Queensland, St Lucia, Queensland 4067, Australia.

Abbreviations used in the text: ARA, acetylene reduction activity; BAPR, benzylaminopurine riboside; cfu, colony forming unit; ESE, effective standard error of the mean; Rif, rifampicin; Sm, streptomycin; TY, tryptone yeast; YEM, yeast extract mannitol.

0885-5765/91/050357+17 $03.00/0 © 1991 Academic Press Limited

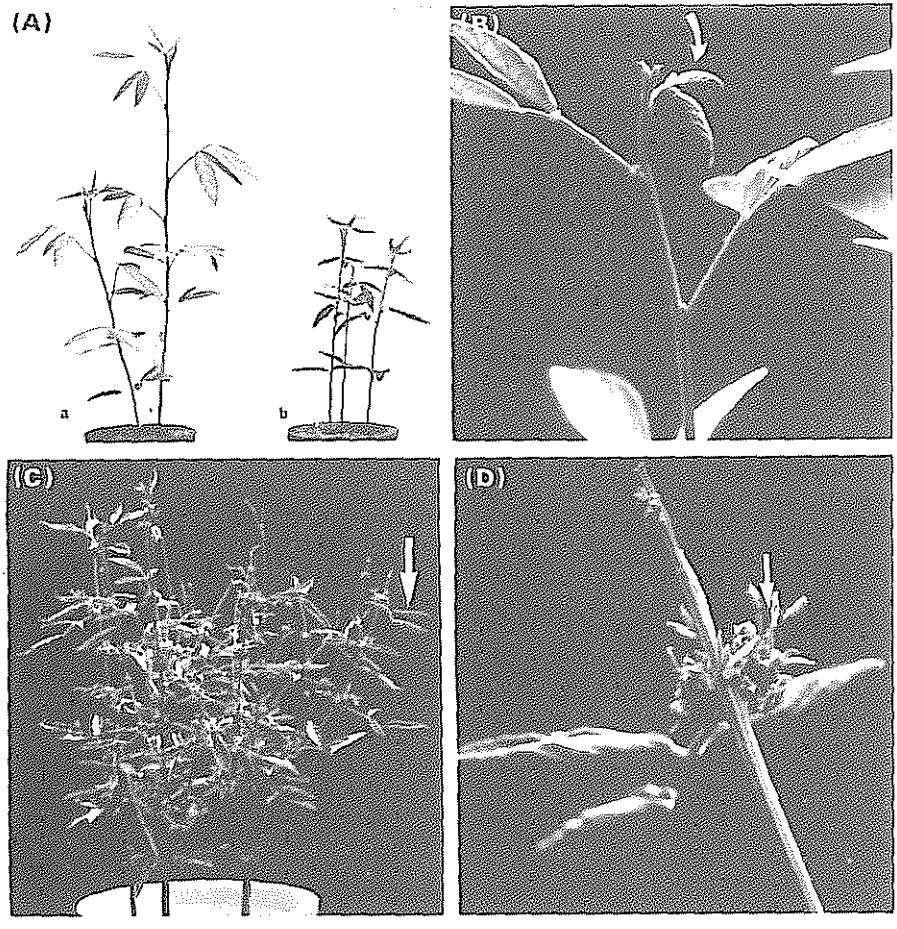

FIG. 1. Leaf curl symptoms in pigeonpea produced by nodulation with *Rhizobium* strain IC3342. (A) Comparison of 50-day-old plants nodulated with (a) normal strain IHP100 and (b) leaf curl-inducing strain IC3342. Nodulation by the strain IC3342 results in leaf-curling, suppression of apical dominance, sprouting of lateral buds, whereas nodulation by the strain IHP100 results in normal development of the plant. (B) *Tip bending*, indicated by an arrow, is the initial symptom observed in a plant nodulated by the strain IC3342 and starts 25–30 days after inoculation. (C) Advanced stages (120 days) of symptoms showing severe leaf-curling, sprouting of several lateral buds (arrow) and stunted growth. (D) A close-up view of the nodal region showing several sprouted buds (arrow).

be an adverse effect. Some *Rhizobium* strains may also produce compounds which affect shoot growth. Interaction between *Glycine max* and particular *Bradyrhizobium japonicum* strains results in a transient chlorosis of the leaves [*21*] induced by the synthesis of rhizobitoxine [*15*] in the root nodules. However, in contrast to *Agrobacterium*-induced tumorigenesis, there is neither evidence for gene transfer from *Rhizobium* to the legume nor for a direct involvement of phytohormones [*32*]. In this context the recently discovered interaction between a *Rhizobium* strain IC3342 and the tropical legume pigeonpea (*Cajanus cajan* (L.) Millsp.) [*16*] is interesting. In addition to apparently

normal nodulation and nitrogen fixation, this interaction leads to a unique developmental change in the plant shoots (hyponasty, curling of the leaves, release from apical dominance, proliferation of lateral buds and stunted growth). The developmental changes depicted in Figs 1(A)–1(D) are similar to those reported by Kumar Rao *et al.* [*16*].

Peach leaf curl caused by the fungus *Taphrina deformans* (Berk.) Tul. and leaf curl symptoms in fasciation or witch's broom caused by *Corynebacterium facians* (Tilford) Dowson have been attributed to hormonal imbalance and specifically to cytokinin over-production [*19*, *27*]. By analogy, the leaf curl symptoms in pigeonpea may be due to hormonal imbalance resulting from nodulation by the *Rhizobium* strain IC3342. If so, the system could serve as a model to study hormone production in otherwise well characterized rhizobia which are amenable to genetic manipulation, and to investigate further the relationship between root derived hormones and shoot development.

In this paper we report on the physiological and genetic characteristics of *Rhizobium* strain IC3342, its host range and its competitiveness in nodule occupancy with other fast and slow growing root-nodule bacteria. We also report on the nature, probable site of production and factors governing the production of the leaf curl inducing principle.

MATERIALS AND METHODS

Bacterial strains, plasmids and host plants

Rhizobium/*Bradyrhizobium* strains, their characteristics and sources are listed in Table 1. Plasmid DNA p5A and pRt587, containing *nifH*/*D* from *Rhizobium* spp. ANU240 (*1*) and *nod* genes from *R. leguminosarum* biovar. *trifolii* ANU843 (*26*), respectively, were kindly supplied by J. M. Watson, CSIRO Division of Plant Industry, Canberra, Australia. Seeds were obtained from J. Brockwell, M. Trinick and M. Peoples, CSIRO, Canberra, Australia.

Bacteriological media and growth conditions

YEM [*35*] and TY media [*2*] were used for *Rhizobium* and *Bradyrhizobium*. Inoculant strains were grown at 29 °C to late log phase as shaken cultures (250 r min^{-1} orbital). Antibiotics (Sm and/or Rif) were used at concentrations of 100 μg ml^{-1}. For bacterial growth measurements 5 ml of a fresh culture (approx. 10^8 cfu ml^{-1}) was inoculated into 100 ml YEM broth in a 500-ml flask with a side-arm. Absorbance ($Klett_{500-570\ nm}$) was measured and viable counts were estimated at various intervals by the drop plate count method [*18*].

Plasmid visualization and location of nif *and* nod *genes*

Plasmids were visualized by a modified Eckhardt method [*22*]. Southern transfer onto a nitrocellulose membrane was carried out as described by Schofield *et al.* [*26*]. A 3·2 kb *Eco*RI fragment cloned (plasmid p5A) from the fast-growing *Rhizobium* ANU240, containing *nifH* and *nifD* [*1*], and a 14 kb *Hin*dIII-*Bam*HI fragment cloned (pRt587) from *R. leguminosarum* biovar. *trifolii* strain ANU843, containing *nod* gene sequences [*26*] were radioactively-labelled with ^{32}P as described previously [*34*]. Hybridizations at high stringency were carried out according to Schofield *et al.* [*26*].

TABLE 1
Rhizobium/Bradyrhizobium *strains used in the study*

Rhizobium/Bradyrhizobium spp.	Strain	Characteristics	Original host	Reference/source
R. leguminosarum				
bivar. *viceae* (Frank) Frank	RL300	wild-type, fast-growing	*Pisum sativum* L.	[13]
bivar. *trifolii* Dangeard	ANU843	wild-type, fast-growing	*Trifolium subterraneum* L.	[6]
bivar. *phaseoli* Dangeard	CC511	wild-type, fast-growing	*P. vulgaris* L.	J. Brockwell
R. meliloti Dangeard	SU47	wild-type, fast growing	*Medicago sativa* L.	J. Vincent
R. loti Jarvis, Pankhurst and Patel	CC829	wild-type, slow-growing	*Lotus pedunculatus* L.	J. Brockwell
	SU343	wild-type, slow-growing	*L. corniculatus* L.	J. Vincent
Bradyrhizobium japonicum (Kirchner) Jordan	CB1809	wild-type, slow-growing	*Glycine max* (L.) Merr.	R. Date
	CB756	wild-type, slow growing	*Macrotyloma africanum* (Brenan ex. Wilczeck) Verdc.	R. Date
	32H1	wild-type, slow-growing	*Crotolaria paulina* Schrank	A. Gibson
	NGR93	wild-type, slow-growing	*Derris elliptica* (Roxb.) Benth.	[28]
Rhizobium spp.	ANU240	a spontaneous Sm^r mutant of NGR234, fast-growing	*Lablab purpureus* (L.) Sweet	[28]
	WE7	wild-type, fast-growing	*Sesbania aculeata* Poir.	ICRISAT
	IC3342	leaf curl-inducing, fast-growing	*Cajanus cajan* (L.) Millsp.	ICRISAT
	IHP100	wild-type, fast-growing	*C. cajan*	ICRISAT
	MNU1	wild-type, fast-growing	*C. cajan*	N. M. Upadhyaya
	ANU1298	a Sm^r Rif^r derivative of IC3342	*C. cajan*	this study
	ANU3027	a plasmid-cured, non-nodulating derivative of ANU1298	*C. cajan*	this study

Plasmid curing

The *Rhizobium* strain ANU1298 (a Sm^r Rif^r derivative of the strain IC3342) was subjected to heat treatment. The strain was streaked onto TY and YEM agar plates and incubated at 37 °C for 7 days. While the strain was still growing, the incubation temperature was increased to 39 °C and incubation continued for a further 7 days. The colonies which survived this heat treatment were again streaked onto a fresh plate and incubated at 40 °C for 10 days and then at 33 °C for 5 days. The colonies growing on this plate were selected and re-streaked, and 20 single colonies tested for the loss of nodulation ability with *Macroptilium atropurpurium* (D.C.) Urb.(siratro) under test-tube growth conditions. The plasmid profile was visualized and probed with radioactively-labelled *nif* and *nod* gene sequences as described earlier.

Plant growth conditions

Plants were raised in a solar energy conserving glasshouse maintained at day temperatures between 25 and 33 °C and a minimum night temperature of 15 °C. Plastic pots (either 10 or 20 cm top diameter) were filled with pre-washed and steamed, sand and vermiculite (60:40, v/v) mixture. One day prior to sowing, pots were saturated with Fahraeus nutrient solution [7] containing 0·9 mM N in the form of potassium nitrate. Seeds were soaked in 3% hydrogen peroxide, washed with water, soaked with 4% sodium hypochlorite and washed several times with water. Seeds with a hard seed coat were scarified with concentrated sulphuric acid for 10 min prior to surface sterilization. Pre-germinated seeds were sown and inoculated with 5 ml of the inoculant cultures (approx. 10^9 cfu ml^{-1}). Nutrient solution was applied two or three times a week. Each treatment was replicated in two or four pots and each experiment was repeated at least once.

A test tube plant growth system also was used with *M. atropurpurium* as the test plant. A growth chamber with a water bath, maintained at 20 °C night (10 h) and 28 °C day (14 h), illuminated by a mercury halide lamp producing a light intensity of approx. 600 $\mu E\ m^{-1}\ s^{-1}$ at plant level was used. Test-tubes (25 × 200 mm) filled to approx. 15 mm with sand + vermiculite (60:40, v/v) saturated with Fahraeus nutrient solution, were capped and autoclaved. Pre-germinated siratro seed was sown in the centre of capped test tubes, incubated for a day in the dark and then in the growth chamber for another day. A sterile cotton plug was then placed just below the tip of the plumule. A sterile Pasteur pipette was pushed tip down through the cotton plug to reach the growth medium level, and this was used to inoculate and subsequently water the test tube. The open pipette end was plugged with sterile cotton wool. The open end of the test tube was covered with aluminium foil after the plant shoot had grown out of the tube to further reduce the chances of cross-contamination.

Host range experiment

Different legume host plants (see Table 2) were grown in two replicate pots inoculated with the strain IC3342. Appropriate *Rhizobium*/*Bradyrhizobium* strains were used as positive controls. Observations were made after 45 days on nodule number, nodule weight, nitrogenase activity [10] and on leaf curling.

TABLE 2

Nodulation, nitrogen-fixation and leaf curl induction in different legume host plants by the Rhizobium *strain IC3342 in comparison with respective nodulating strains of those host plants*

Host plant	Cultivar	Strain	Plant No.	Shoot d. wt (g)	Nodule No.	Nodule d. wt (mg)	ARA[a]	Leaf curl
Lathyrus sativus L.	CPI31616	IC3342	2	0·55	0	0	0	–
		RL300	2	0·68	> 200	148	352	–
Vicia faba L.	Fiord	IC3342	2	1·08	0	0	0	–
		RL300	2	2·52	> 200	160	1088	–
Pisum sativum L.	Dunfield	IC3342	2	0·48	0	0	0	–
		RL300	2	1·21	> 200	73	520	–
Cicer arietinum L.	Tyson Desi	IC3342	2	0·43	0	0	0	–
		RL300	2	0·40	0	0	0	–
Trifolium pratense L.	Redqnin	IC3342	8	0·07	0	0	0	–
		ANU843	8	0·11	63	32	31	–
Phaseolus vulgaris L.	Redland Pioneer	IC3342	2	1·42	> 200	43	38	–
		CC511	2	1·92	> 200	168	736	–
Medicago sativa L.	Hunter River	IC3342	4	0·05	0	0	0	–
		SU47	4	0·29	110	82	158	
Lotus major Scop.	—	IC3342	2	0·01	0	0	0	–
		CC829	2	0·01	10	3	0	–
L. corniculatus L.	—	IC3342	2	0·01	0	0	0	–
		SU343	2	0·01	4	3	12	–
Glycine max (L.) Merr.	Forrest	IC3342	2	1·51	0	0	0	–
		CB1809	2	2·15	7	35	164	–
Vigna mungo (L.) Hepper	Regur	IC3342	2	0·20	54	45	19	+
		CB756	2	0·17	5	7	42	–
V. radiata (L.) Wilczek	Berkum	IC3342	2	0·93	166	98	109	+
		CB756	2	0·41	5	29	6	–
V. unguiculata (L.) Walp.	Vita 3	IC3342	2	0·85	88	183	116	+
		CB756	2	0·61	41	85	66	–
Arachis hypogaea (L.)	A72/28	IC3342	1	1·00	0	0	0	–
		CB1809	1	1·10	25	46	352	–
Leucaena leucocephala (Lam.) deWit.	Cunninghar	IC3342	3	0·20	12	16	13	–
		NGR93	3	0·25	44	25	278	–
Desmodium intortum Urd.	Greenleaf	IC3342	14	0·04	47	18	2	–
		IHP100	14	0·42	151	50	612	–
Cajanus cajan (L.) Millsp.	Hunt	IC3342	2	0·59	81	105	376	+
		IHP100	2	0·64	65	110	428	–
Sesbania rostrata Brem. Oberm.	—	IC3342	3	0·20	103	23	5	–
		WE7	3	0·19	79	19	1	–
Macroptilium (Siratro) atropurpurium (D.C.) Urb.	—	IC3342	3	0·13	92	28	61	+
		IHP100	3	0·26	34	35	70	–

[a] Acetylene reduction activity of excised roots of 45-day-old plants, following 1 h incubation with 15% C_2H_2.

ARA expressed in nmol C_2H_4 per pot per hour.

Values are average of two replicate pots.

Mixed inoculation experiment

Strain ANU1298 was used alone and in combination with a fast-growing *Rhizobium* MNU1 or a slow-growing *B. japonicum* 32H1. Inoculations (approx. 10^9 cfu per pot) were done immediately after sowing pigeonpea. Each treatment was replicated in four pots. Nodules were harvested after 45 days, washed in water, and then surface-sterilized individually with the apparatus designed by Gault *et al.* [8]. The nodules in

this system were first rinsed in 95% (v/v) ethanol for 15 s then with 0·1% (w/v) mercuric chloride for 30 s followed by five to six water washes, and finally rinsed in protoplast dilution buffer (0·25 M sorbitol, 0·25 M mannitol, 2 mM K_2HPO_4, 2 mM $CaCl_2$ pH 5·8). Nodule occupants were isolated on Congo red YEM and YEM Sm Rif agar media. Strains were identified according to their growth rates and growth patterns on the two media.

Nitrate effect experiment

Nitrogen levels of 0, 3·6, 7·2, 14·4 and 36 mM were supplied to pot-grown pigeonpea through the nutrient solution in the form of potassium nitrate. Nitrate addition was started at sowing in one set of treatments and 10 days after sowing, i.e. after nodule initiation, in another set (each with four replicate pots). Sufficient nutrient solution containing KNO_3 at the above concentrations, was added to the pots to flush the sand + vermiculite with the new medium. Nodule number, nodule dry weight, shoot dry weight, nitrogenase activity and leaf curling were recorded on day 33. Nitrogenase activity, as acetylene reducing activity (ARA) in the nodules, was measured on decapitated nodulated roots [*10*].

Grafting experiment

Plants were grown in separate pots, and spliced approach grafting [*11*] was performed with two stems of the same size. In this method, a slice of bark and wood, 2·5 cm long and 0·5 mm wide, was cut from both stems keeping the slice as flat as possible. The two cut surfaces were then bound tightly together with grafting tape.

Xylem exudate, plant extracts, bacterial extracts and cytokinin feeding experiments

Xylem exudate, leaf extract and nodule extract from plants nodulated by strain IC3342 and a normal strain IHP100, and cell-free extracts of stationary phase cultures of these strains were fed, either by applying to the growth medium, or by a wick method to test tube-grown healthy pigeonpea plants.

Pot-grown plants, 35–40 days old, were cut 3 cm above the ground level. A flexible silicone rubber tube was pushed over the stump of the stem to serve as a reservoir to collect the bleeding sap. Sap collection began at 11 a.m. on a bright day and was completed within 1 h of cutting the stem. The sap was stored at −20 °C in a vial with twice its volume of methanol containing 5% (v/v) acetic acid.

Fresh leaves (10 g) or fresh nodules (5 g) were macerated in 100 ml of phosphate buffer (100 mM, pH 6·5) and kept at 4 °C overnight to allow debris to settle. The supernatant was decanted carefully and passed through a 0·22 μm membrane filter. The filtrate volume was reduced to 10 ml by vacuum evaporation (45 °C). To prepare cell-free culture extracts, 100 ml of stationary phase culture in YEM broth was clarified by centrifugation, passed through a 0·22 μm membrane filter and vacuum evaporated to 10 ml.

A wick-feeding method [*12*] was employed for feeding the sap or the cytokinin BAPR. A hole was made through the xylem and pith of the stem between the second and third youngest leaves of a 20–25-day-old plant and, using a needle, a doubled cotton thread was passed through the hole. The other end of the wick was immersed

in a small vial containing the sap or cytokinin. A fresh hole was made daily, just above the previous one, and the procedure repeated for 2 or 3 days.

Leaf and nodule extracts, cell-free culture extracts and different concentrations of the cytokinin BAPR (0, 2·8 μM, 28 μM, 280 μM, 2·8 mM and 28 mM) were fed to the growth medium in the test tube. This application was done either at the time of sowing or to 10-day-old seedlings. The effects were recorded after 25 days.

RESULTS

Growth characteristics

The leaf curl inducing strain IC3342 was compared with the well-characterized, fast-growing *Rhizobium* strain ANU240 (a Sm^r NGR234). Both strains grew well in YEM agar medium and did not absorb Congo red dye on agar plates (data not shown). Both the strains produced copious amounts of extracellular polysaccharides. The strain IC3342 had less mucoid growth than ANU240 as measured by the absorbance ($Klett_{500-570\ nm}$). Broth culture medium (YEM) of strains IC3342 and ANU240 with approx. 10^9 cfu had the Klett reading of 225 and 360, respectively. The growth rate of strain IC3342 in this medium was similar to that of ANU240. In a shaken culture (29 °C), the strain IC3342 had a generation time of 3·47 h and a growth constant (k) of 0·29 at log phase. Under similar conditions, strain ANU240 had a generation time of 3·22 h and a k value of 0·31.

Plasmid profile and location of nif *and* nod *genes*

Horizontal gel electrophoresis of the cell lysates prepared by a modified Eckhardt method showed three megaplasmids in strain IC3342, the largest of which was detectable only when the strain had been repeatedly subcultured two to three times in YEM [Fig. 2(A)]. The two largest megaplasmids were similar in size to those in strain ANU240, which have estimated molecular weights of $> 450 \times 10^6$ and 310×10^6 [*22*]. The estimated molecular weight of the third plasmid was 200×10^6.

Both ANU843 (*R. leguminosarum* biovar. *trifolii*) *nod* and ANU240 *nif* gene probes apparently hybridized to the same megaplasmid [Fig. 2(B) and 2(C)]. In a separate experiment with extended electrophoresis, the second largest plasmid hybridized to the *nif* and *nod* gene probes (data not shown) indicating it to be the symbiotic (Sym) plasmid. When an *Eco*RI digest of total DNA was hybridized with *nod* gene sequences, seven hybridizing fragments were visualized [Fig. 3(A)]. Reprobing (after de-probing) of the same blot with *nif* sequences showed two similarly-strong hybridizing fragments of 9 kb and 2·5 kb [Fig. 3(B)]. These data suggest that in IC3342 the symbiotic genes are plasmid borne, reiterated and highly conserved, especially among strains ANU240 and IC3342.

Effect of plasmid curing on nodulation and leaf curling

Tests for the effect of plasmid-curing on symptom development were carried out in an axenic test tube growth system using siratro as the test host plant. Of the 20 heat-treated isolates tested, only ANU3027 (ANU1298 Nod^-) did not nodulate siratro or induce leaf curling. Under the conditions used for visualizing plasmids, only the

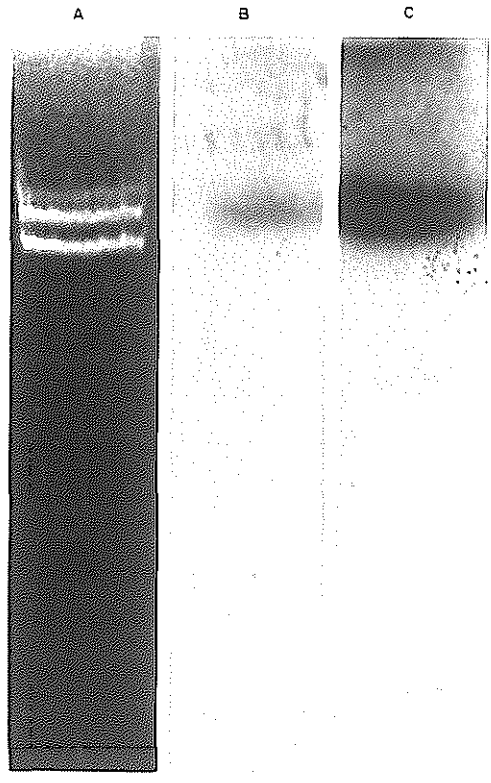

FIG. 2. Plasmid visualization by horizontal gel electrophoresis and hybridization with *nif* and *nod* gene probes. (A) Megaplasmids present in the strain IC3342 visualized by a modified Eckhardt method [22]. Autoradiograms of a Southern blot of these plasmids hybridized with ^{32}P-labelled *nod* gene (pRt587) sequences from ANU843 (B) and *nif* gene (p5A) sequences from ANU240 (C).

intermediate-sized plasmid was found in this strain and the other two could not be visualized. The *nif* and *nod* gene probes hybridized to this single plasmid suggesting that it was the Sym plasmid (data not shown).

Host range

The strain IC3342 nodulated *Phaseolus vulgaris*, *Vigna mungo*, *V. radiata*, *V. unguiculata*, *Desmodium intortum*, *Cajanus cajan*, *Leucaena leucocephala*, *Sesbania rostrata* and *Macroptilium atropurpurium* (Table 2). However, only nodules from *V. mungo*, *V. radiata*, *V. unguiculata*, *C. cajan* and *M. atropurpurium* were able to fix nitrogen, as evidenced by nitrogenase activity (ARA) and only these plants produced leaf curl symptoms. Although IC3342 induced numerous nodules in *P. vulgaris*, as did the homologous strain CC511, the AR activity was markedly less compared to that produced by CC511 nodulation, and no leaf curling symptoms were observed. Plants from other cross-inoculation groups were not nodulated by strain IC3342 and were symptomless. All plants except *Cicer arietinum*

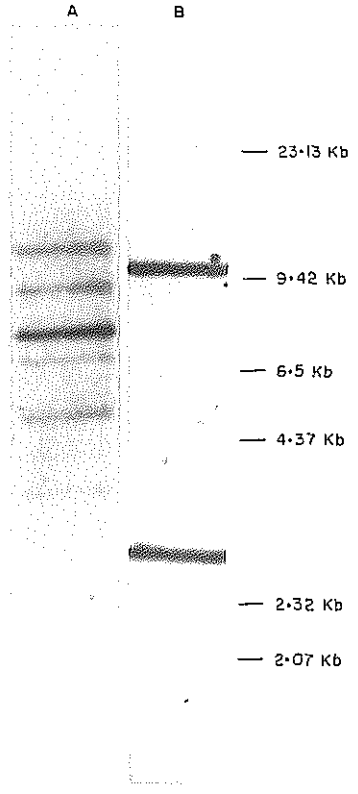

FIG. 3. Hybridization of IC3342 DNA with *nif* and *nod* gene probes. Autoradiogram of Southern blots of *Eco*RI digested genomic DNA from the strain IC3342 hybridized with ^{32}P-labelled *nod* gene sequences from ANU843 (A) and *nif* gene sequences from ANU240 (B).

were effectively nodulated by homologous strains of rhizobia. Pigeonpea (*C. cajan*) was the first plant to have leaf-tip bending which was observed within 24 days of inoculation. Pigeonpea also had the highest ARA and the most severe leaf curl symptoms among different host plants.

Competitiveness

The Sm^{r}Ril^r derivative strain of IC3342 (ANU1298) was as effective in nitrogen-fixation as the parent (data nor shown). It formed similar numbers of nodules on pigeonpea and siratro. The fast-growing *Rhizobium* strain MNU1 and the slow-growing strain 32H1 were tested for competitiveness with ANU1298. Nodule representation by strain ANU1298 is presented in Table 3. For plants inoculated with ANU1298 alone, 90% of the nodules were found to contain the inoculum strain. The recovery of the strain ANU1298 when competing with strain MNU1, was only 11% and 9% with strain 32H1. However, most of the initially-formed (mature) nodules were occupied by strain ANU1298 (data not shown). This level of nodule occupancy was sufficient to produce the leaf curl syndrome in these doubly-inoculated plants.

TABLE 3

Effect of mixed inoculation of the strain ANU1298 with fast-growing and slow-growing root-nodule bacterial strains on nodule occupancy and leaf curl induction

Inoculum	Nodule No.	Nodules formed by ANU1298	% occupancy by ANU1298	Leaf curl
ANU1298	110	99	90	+
ANU1298 + MNU1	136	15	11	+
ANU1298 + 32H1	124	11	9	+
ESE	3·7	4·2		

ESE = effective standard error of the mean.
Values are average of four replicate plants harvested 45 days after sowing.

TABLE 4

Effect of inorganic nitrogen on nodulation, nitrogen fixation and leaf curling in pigeonpea produced by the strain IC3342

Treatment (N in mM)[a]	Nodule No.	Nodule d. wt (mg)	ARA[b]	Shoot d. wt (g)	Leaf curl
Started at sowing					
0	93	57	127	0·24	+
3·6	56	108	364	0·93	+
7·2	37	47	101	1·25	−
14·4	27	18	19	1·24	−
36·0	7	5	1	1·04	−
Started after 10 d					
0	72	45	95	0·21	+
3·2	71	88	380	0·74	+
7·2	72	64	176	1·05	−
14·4	54	32	50	1·09	−
36·0	76	20	2	0·73	−
ESE	8·2	8·4	50·4	0·090	

[a] Plants were supplied with nutrient solution ± N in the form of KNO_3.
[b] Acetylene reduction activity (expressed as nmol C_2H_4 per plant per h^{-1}) of excised roots following 1-h incubation in 15% C_2H_2.
Values are average of four replicate plants harvested 33 days after sowing.
ESE = Effective standard error of the mean.

Effect of nitrate

Among pigeonpea plants inoculated with strain IC3342 and different levels of nitrate, nodule number decreased when increasing nitrate levels were supplied from the day of sowing (Table 4). Plants receiving no nitrogen formed an average of 93 nodules per plant, while those receiving 36 mM N had an average of only seven nodules per plant. However, when the nitrogen treatment was delayed for 10 days, nitrate level had no significant effect on nodule number. Nodule weight also decreased with increasing

FIG. 4. The lower portion of a healthy plant originally grafted on to a leaf curl plant, then separated from the leaf curl plant after 28 days and allowed to grow for another 20 days. (a) Healthy stalk, (b) region of graft, (c) shoots developed after graft union and (d) shoots developed after graft separation.

levels of nitrate beyond 3·6 mM. The ARA also followed the same trend with most activity for plants receiving 3·6 mM N. For both times of nitrate application there was no ARA at the 36 mM N level. Interestingly, plants receiving 0 and 3·6 mM N developed leaf curl symptoms in both nitrogen application regimes while larger amounts of nitrate completely prevented symptom expression.

Grafting experiments

Two 30-day-old pigeonpea plants, one nodulated by leaf curl-inducing strain IC3342 and the other nodulated by the normal strain IHP100, were approach grafted near the second nodal region. Symptoms started to develop on the growing shoots of the IHP100-nodulated plant within 8 days. After a further 20 days, symptoms also developed on shoots below the region of the graft. At this stage, the plants were separated and allowed to grow for another 20 days. The leaf curl symptoms persisted in the old leaves (Fig. 4). However, new shoots developed without the leaf curl symptoms. This indicated that the leaf curl-inducing principle was produced in the roots/nodules and was translocated to the growing shoots. These results suggested that the effect of the inducing principle was on the meristematic tissue and that the effect was irreversible.

Feeding experiments

Healthy pigeonpea plants were fed with leaf extract, nodule extract and xylem exudate (sap) from IC3342-nodulated pigeonpea plants and cell-free (IC3342) culture extracts to study their ability to induce leaf curling. Extracts from plants nodulated by a normal strain IHP100 and cell-free IHP100 culture extracts were used as controls. Initial symptoms of leaf curling, i.e, tip bending, were observed in healthy plants fed by the wick method with a 10 fold concentrated xylem sap collected from a leaf curled plant. Tip bending was also observed in plants fed through the rooting medium with leaf extract from leaf curl plants, while other treatments failed. No symptom was observed in plants fed with xylem sap, or various extracts from IHP100 nodulated plants or IHP100 culture media.

The cytokinin BAPR was also supplied to the roots of 10-day-old pigeonpea seedlings, grown in sand+vermiculite at various concentrations. The BAPR at concentrations less than 2·8 μM had no visual effect. Hyponasty and root volume reduction were observed in plants which received 28 μM BAPR and the effects were severe at higher concentrations. At concentrations greater than 2·8 mM, lateral buds developed indicating a release from apical dominance. However, BAPR did not induce the leaf curling or irregular leaf surface development seen in plants nodulated by the strain IC3342.

DISCUSSION

An unusual root nodule bacterium IC3342, which causes leaf curl syndrome in the tropical legume pigeonpea, was further characterized. The nodulation host range, physiological and genetic characters of the strain IC3342 are consistent with those of the fast-growing *Rhizobium* ANU240 (NG234). Generally, *Rhizobium* strains nodulating tropical legumes are slow-growing and have been classified recently into the new genus *Bradyrhizobium* [*14*]. However, few fast-growing rhizobia such as NGR234, have been isolated from tropical legumes [*28*] which have a wide host range [*17*, *28*]. Fast-growing strain IC3342 which was originally isolated from a tropical legume, pigeonpea [*16*], effectively nodulated pigeonpea, species of *Vigna* and siratro, but ineffectively nodulated *P. vulgaris* (compared to its nodulation with the homologous strain CC511), *L. leucocephala* and *S. rostrata*. However, these legumes were reported to be effectively nodulated by strain ANU240 [*17*, *28*].

Similar plasmid profiles, Sym plasmid and *nifH/D* gene reiterations reinforced the close similarly between strains IC3342 and ANU240. Even though the significance of more than one copy of a particular gene is not known, such reiteration of genes has been reported in *Rhizobium* strains and, in some cases, both have been shown to be functional [*20*, *23*, *24*]. Gene reiterations have also been reported in *R. leguminosarum* biovar. *trifolii nod* genes [*26*]. Hybridization with *R. leguminosarum* biovar. *trifolii* (ANU843) *nod* genes suggested similar reiterations in the strain IC3342.

The leaf curl inducing strain competed poorly in forming nodules with the two other strains of the cowpea group rhizobia. Trinick *et al.* [*29*] also reported a poor competition of strain NGR234 against slow-growing rhizobia under warm (30 °C) plant growth conditions. This might explain why leaf curl symptoms are not observed

under tropical field conditions [*16*]. However, the strain used was a spontaneous mutant (Sm^r Rif^r) of the original strain IC3342, and its competitiveness in nodule formation is not known. In pot trials, nodule occupancy of about 10% was sufficient to produce leaf curl symptoms. We did not determine whether young plants with mixed nodule population, but with leaf curl symptoms, would recover from the symptoms.

Grafting studies indicated that the curl-inducing principle is a systemic factor produced in the roots and/or nodules and translocated to the developing shoots to produce the symptoms. A continuous flow of this systemic factor is essential for the continuous manifestation of the symptoms. This suggests that the systemic factor is a hormone-like compound rather than a virus or bacterium. The normal development of the shoots emerging on the healthy stalk after graft separation could have resulted from metabolic inactivation and/or dilution of the inducing principle. Furthermore, only the meristematic tissues were affected and not fully-developed leaf tissue.

Feeding experiments revealed the presence of the inducing principle in the xylem sap and also in leaf extracts which is further evidence for its systemic nature. Exogenous application of the cytokinin BAPR could induce only partial symptoms compared to those produce by IC3342 nodulation. Possible differences in the effects of endogenous production and exogenous application could explain this. Feeding experiments did not indicate the presence of the systemic factor in the nodule extracts or in cell-free culture extracts of *Rhizobium* strain IC3342 but, as these were crude extracts, the results are not conclusive. It is possible also that the inducing principle is produced only after the *Rhizobium*–plant symbiosis is established.

Examination of a non-nodulating derivative strain, obtained after growth at 40 °C, demonstrated the loss of at least the smallest megaplasmid. The loss of the largest megaplasmid is questionable because of the difficulties in reproducibly visualizing it with the method employed. Hybridization of *nif* and *nod* gene probes to the remaining plasmid of the plasmid-cured strain suggested that this was the Sym plasmid. The lack of nodulation could also result from either an internal deletion in the Sym plasmid in the region which affects nodulation, or the cured plasmid(s) may also carry determinants necessary for nodulation, in addition to those carried on the Sym plasmid. Deletions of *nod-nif* sequences are common among strains surviving growth at elevated temperatures [*6*, *9*] and repeated sequences of DNA found in *Rhizobium* [*33*] are implicated in such deletions. Furthermore, mutations other than in the *nif* structural genes have been mapped on the indigenous plasmid and the chromosome in *Rhizobium* strains [*3*, *25*]. Therefore it is possible that the cured plasmid(s) may contain nodulation determinants. However, the leaf curl symptoms appear to depend on nodule formation.

Leaf curl induction was observed only in those hosts where there was effective nodulation, suggesting that the development of functional nodules was necessary for the production of the leaf curl-inducing principle. Even though the nodules formed in *P. vulgaris* exhibited some AR activity, it was markedly less compared to those in nodules formed by strain CC511. Thus, the effectiveness of strain IC3342 in *P. vulgaris* was not sufficient to produce leaf curl symptoms. This was confirmed by studies with

nitrate. Leaf curling was prevented when nitrate addition allowed nodule formation, but not nitrogenase activity. The delayed addition of nitrate allowed nodule formation but inhibited their proper growth and development. When a low level of nitrate (3·6 mM) stimulated nodule development, leaf curl symptoms also developed. However, plants receiving 7·2 mM nitrogen had effective nodules but no leaf curl symptoms were observed. This could be due to delayed development of nodules in the presence of 7·2 mM N. Although nodules had substantial nitrogenase activity by day 35, they might not have had time to induce a sufficiently large amount or concentration of the leaf curl inducing principle to induce symptoms. The enhanced growth of shoots resulting from the 7·2 mM N treatment might also have effectively diluted the curl inducing principle below the critical level necessary for symptom induction.

The results of this study indicate that a curl-inducing principle is produced during effective nodulation by strain 1C3342 and that this principle is translocated to the shoots to produce the symptoms. In other studies, we have shown that *Rhizobium*-induced leaf curl syndrome is a multi-gene phenomenon (to be published) and provide evidence for the involvement of cytokinins in the process [*30*, *31*]. Thus, the system has the potential to serve as a model for the study of hormone action in *Rhizobium*–legume interactions and, more importantly, in other leaf curl type diseases.

We thank Drs M. Trinick, M. Peoples and J. Brockwell, CSIRO Division of Plant Industry, Australia for providing *Rhizobium* strains and legume seeds. We thank Dr J. M. Watson, CSIRO Division of Plant Industry, Dr J. R. L. Walker and Dr P. Broady, Plant and Microbial Sciences, University of Canterbury, New Zealand for their comments on the manuscript.

REFERENCES

1. Badenoch-Jones, J., Holton, T. A., Morrison, C. M., Scott, K. F. & Shine, J. (1989). Structural and functional analysis of nitrogenase genes from the broad-host-range *Rhizobium* strain ANU240. *Gene* **77**, 141–153.
2. Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology* **84**, 188–198.
3. Beringer, J. E., Benyon, J. L., Buchanan-Wollston, A. V. & Johnston, A. W. B. (1977). The isolation of conditional ineffective mutants of *Rhizobium leguminosarum*. *Journal of General Microbiology* **98**, 339–343.
4. Beringer, J. E., Brewin, J. L., Buchanan-Wollaston, A. W. B., Schulman, H. M. & Hopwood, D. A. (1979). The *Rhizobium*-legume symbiosis. *Proceedings of the Royal Society of London, B* **204**, 219–233.
5. Dart, P. J. (1988). Infection of legumes by *Rhizobium*. In *Microbes in Action*, Ed. by I. Kennedy & W. Murrell, pp. 35–52. John Wiley, London.
6. Djordjevic, M. A., Zurkowski, W. & Rolfe, B. G. (1982). Plasmids and stability of symbiotic properties of *Rhizobium trifolii*. *Journal of Bacteriology* **151**, 560–568.
7. Fahraeus, G. (1957). The infection of clover root hairs by nodule bacteria, studied by a simple glass slide technique. *Journal of General Microbiology* **16**, 374–381.
8. Gault, R. R., Byrne, P. T. & Brockwell, J. (1973). Apparatus for surface sterilization of individual legume root nodules. In *Laboratory Practices*, Vol 22, Commonwealth Scientific and Industrial Research Organization, pp 292–293. CSIRO, Canberra.

9. Hann, M., Meyer, L., Studer, D., Regensburger, B. & Hennecke, H. (1984). Insertion and deletion mutations within the *nif* region of *Rhizobium japonicum*. *Plant Molecular Biology* **3**, 159–168.
10. Hardy, R. W. F., Burns, R. C. & Holsten, R. D. (1973). Application of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biology and Biochemistry* **5**, 47–81.
11. Hartmann, H. T. & Kester, D. E. (1983). *Plant Propagation Principles and Practices*, 4th edn. Prentice-Hall, New Jersey.
12. Jameson, P. E., Letham, D. S., Zhang, R., Parker, C. W. & Badenoch-Jones, J. (1987). Cytokinin translocation and metabolism in lupin species. I. Zeatin riboside introduced into the xylem at the base of *Lupinus angustifolius* stems. *Australian Journal of Plant Physiology* **14**, 695–718.
13. Johnston, A. W. B. & Beringer, J. E. (1975). Identification of the *Rhizobium* strains in pea root nodules using genetic markers. *Journal of General Microbiology* **87**, 343–350.
14. Jordan, D. C. (1982). Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing root nodule bacteria from leguminous plants. *International Journal of Systematic Bacteriology* **32**, 136–139.
15. Keith, D. D., de Bernardo, S. & Weigels, M. (1975). The absolute configuration of rhizobitoxine. *Tetrahedron* **31**, 2629–2632.
16. Kumar Rao, J. V. D. K., Dart, P. J. & Kiran, U. M. (1984). *Rhizobium* induced leaf roll in pigeonpea [*Cajanus cajan* (L) Millsp.]. *Soil Biology and Biochemistry* **16**, 89–91.
17. Lewin, A., Rosenberg, C., Stanley, J., Dowling, D. N., Manen, J. F., Debelle, F. & Broughton, W. J. (1987). Multiple host-specificity loci in the broad host-range *Rhizobium* NGR234. In *Molecular Genetics of Plant–Microbe Interactions*, Ed. by D. P. S. Verma & N. Brisson, pp. 11–13. Martinus Nijhoff, Boston.
18. Miles, A. A. & Mishra, S. S. (1938). The stimulation of the bactericidal power of blood. *Journal of Hygiene, Cambridge* **38**, 732–749.
19. Murai, N., Skoog F., Doyle M. E. & Hanson R. S. (1980). Relationship between cytokinin production, presence of plasmids, and fasciation caused by strains of *Corynebacterium fasciens*. *Proceedings of the National Academy of Sciences of the U.S.A.* **77**, 619–623.
20. Norel, F. & Elmerich, C. (1987). Nucleotide sequence and functional analysis of the two *nifH* copies of *Rhizobium* ORS571. *Journal of General Microbiology* **133**, 1563–1576.
21. Owens, L. D. & Wright A. A. (1965). Rhizobial-induced chlorosis in soybeans: isolation, production in nodules, and varietal specificity of the toxin. *Plant Physiology* **40**, 927–930.
22. Plazinski, J., Cen, Y. H. & Rolfe, B. G. (1985). General method for the identification of plasmid species in fast-growing soil microorganisms. *Applied and Environmental Biology* **49**, 1001–1003.
23. Prakash, R. K. & Atherly, A. G. (1984). Reiteration of genes involved in symbiotic nitrogen fixation by fast-growing *Rhizobium japonicum*. *Journal of Bacteriology* **160**, 785–787.
24. Quinto, C., De La Vega, H., Flores, M., Leemans, J., Cevallos, M. A., Pardo, M. A., Azpiroz, R., De Lourdes Gerard, M., Calva, E. & Palacios, R. (1985). Nitrogenase reductase: a functional multi gene family in *Rhizobium phaseoli*. *Proceedings of the National Academy of Sciences of the U.S.A.* **82**, 1170–1174.
25. Ruvkun, G. B., Sundaresan, V. & Ausubel, F. M. (1982). Directed transposon *Tn*5 mutagenesis and complementation analysis of *Rhizobium meliloti* symbiotic nitrogen fixation genes. *Cell* **29**, 551–559.
26. Schofield, P. R., Ridge, R. W., Djordjevic, M. A., Rolfe, B. G., Shine, J. & Watson, J. M. (1984). Host-specific nodulation is encoded on a 14kb DNA fragment in *Rhizobium trifolii*. *Plant Molecular Biology* **3**, 3–11.
27. Sziraki, L., Balazs, E. & Kiraly, Z. (1975). Increased levels of cytokinin and indole acetic acid in peach leaves infected with *Taphrina deformans*. *Physiological Plant Pathology* **5**, 45–50.
28. Trinick, M. J. (1980). Relationship amongst the fast-growing rhizobia of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. *Journal of Applied Bacteriology* **49**, 39–53.
29. Trinick, M. J., Rhodes, R. L. & Galbraith, J. H. (1983). Competition between fast- and slow-growing tropical legume rhizobia for nodulation of *Vigna unguiculata*. *Plant and Soil* **73**, 105–115.
30. Upadhyaya, N. M., Letham, D. S., Parker, C. W., Hocart, C. H. & Dart, P. J. (1991). Do rhizobia produce cytokinins? *Biochemistry International* **24**, (in press).
31. Upadhyaya, N. M., Parker, C. W., Letham, D. S., Scott, K. F. & Dart, P. J. (1991). Evidence for cytokinin involvement in *Rhizobium* (IC3342)-induced leaf curl syndrome of pigeonpea (*Cajanus cajan* Millsp.) *Plant Physiology* **95**, 109–125
32. Vange, C. P., Boylan, K. L. M. & Stade, S. (1987). Host plant determinants of legume nodule function: similarities to plant disease situations. In *Molecular Determinants of Plant Diseases*, Ed. by S. Nishimura, pp. 271–287. Springer, Tokyo.

33. Watson, J. M. & Schofield, P. R. (1986). Species-specific, symbiotic plasmid located repeated DNA sequences in *Rhizobium trifolii*. *Molecular and General Genetics* **199**, 279–289.
34. Whitfield, P. L., Seeburg, P. H. & Shine, J. (1982). The human proopiomelanocortin gene: organization, sequence, and interspersion with repetitive DNA. *DNA* **1**, 133–143.
35. Zurkowski, W. & Lorkiewicz, Z. (1978). Effective method for the isolation of non-nodulating mutants of *Rhizobium trifolii*. *Genetical Research* **32**, 311–314.