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Cereal Symbiotic Nitrogen Fixation – The Interaction of Rhizobia with Cereals for Symbiotic Nitrogen Fixation

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Abstract. The introduction of symbiotic biological nitrogen fixation into the major non-legume crops of the world would be one of the most significant contributions that biotechnology could make to agricultural sustainability. With this objective in mind we have undertaken a study of the interaction of rhizobia with the root systems of cereals to determine whether an intracellular symbiotic nitrogen fixing association with rhizobia could be achieved of benefit to plant growth and development.
We have previously demonstrated that some naturally occurring rhizobia, such as those isolated from root nodules of non-legume *Parasponia* species and from stem nodules of tropical *Aeschynomene* legume species, are able to enter the root systems of maize, rice and wheat by 'crack entry'. This occurs where lateral roots emerge through the root cortex, resulting in the penetration of rhizobia both between and into cells of the cortex, particularly in the cortex of emerging lateral roots.
Recently, we have interacted oxygen tolerant *Azorhizobium caulinodans* ORS571 (kindly donated by Dr J K Ladha, IRRI) isolated from stem nodules of the tropical legume *Sesbania rostrata* with the root systems of rice and wheat. We have found that intracellular invasion of cells of the cortex of roots of both rice (IR42 and Lemont) and wheat (Wembley) results in plants that are active in nitrogen fixation as determined using acetylene reduction assays. Currently, we are quantifying, using both acetylene reduction and 15N dilution assays, the extent to which this nitrogen fixation is due to symbiotic intracellular rhizobia, and whether it will be beneficial to the growth and development of rice and wheat plants.

Key words. Cereals, nodules, nitrogen fixation.

Introduction

In symbiotic associations between soil bacteria *(Rhizobium, Bradyrhizobium* and *Azorhizobium)* and the members of the plant family Leguminosae, the rhizobia infect the root via either the root hairs or by crack entry caused by emerging lateral or adventitious roots and induce the formation of morphologically-defined structures called nodules. The only non-legumes known to naturally form nodules with either *Rhizobium* or *Bradyrhizobium* belong to the genus *Parasponia* which are infected close behind the growing tip of the root by erosion of the surface of epidermal cells. Infection requires the initiation of cell division followed by the formation of infection threads from bacterial colonies within the root cortex and the induction of a lateral root below the site of cell division. The final structure is a lateral root swollen on either side of the central vascular tissue by cortical cells filled with infection threads containing bacteria actively engaged in nitrogen fixation. An important finding has been that some strains of rhizobia can nodulate both legumes and *Parasponia* (1).

It was found that treatment of seedling roots with a mixture of cell wall degrading enzymes, cellulase and pectolyase, could rapidly remove the cell wall at the tip of root hairs of a wide range of crop plants, both legumes and non-legumes, including cereals (2). It was also found that treatment of root hairs of white clover treated with cell wall degrading enzymes (cellulase-pectolyase) removed a barrier to *Rhizobium*-host specificity and enabled normally excluded *R. loti* bacteria to infect plants and form nitrogen fixing nodules. Enzyme treatment was thus shown to remove a barrier to infection and also to host specific regulation of nitrogen fixation (3). Nodulation of non-legumes was also achieved at low frequency by applying rhizobia to enzyme-treated roots of both rice and wheat and oilseed rape (4). The internal struc-

ture of these nodular structures indicated that they were outgrowths from the root cortex with little tissue organisation. They were not of lateral root origin and whilst rhizobia were present both between and within cells no significant levels of nitrogen fixation were detected using acetylene reduction assays.

Significantly it was observed that nodulation of oilseed rape occurred in the absence of enzyme treatment when the roots of oilseed rape seedlings were inoculated with *Bradyrhizobium* CP283 naturally nodulating non-legume *Parasponia* spp. (5). this finding led to extensive investigations to determine whether similar nodules of lateral root origin could be induced without any enzyme treatment on cereals by rhizobia naturally nodulating Parasponia spp. and by rhizobia naturally stem nodulating tropical legumes such as *Aeschynomene* and *Sesbania* spp. Assessments were also undertaken of the details of the interaction pathways, and of the extent to which such nodules would be active in symbiotic nitrogen fixation.

I – Materials and Methods

1. Inoculations with rhizobia and growth of plants

Seeds of maize (variety John Innes hybrid) were surface sterilised and initially germinated in 14 cm Petri dishes before transfer to jars and incubated at 25˚C, 16h day length on a medium (RH) lacking fixed nitrogen and inoculated with either Rhizobium ORS310 naturally nodulating Aeschynomene spp. or *Bradyrhizobium* CP283 naturally nodulating non-legume Parasponia spp. These procedures, and staining with tetrazolium red and light microscopic procedures were as previously described (Ref 6). *Azorhizobium caulinodans* (ORS571) was grown for three days on TGYE medium, solidified with 0.8% agar at 27˚C in the dark; a loopful of bacteria was subsequently transferred to 50 ml TGYE broth for 3 days. Seeds of rice (varieties Lemont and IR42) were manually dehusked and sterilised in 30% bleach for 60 min. followed by 6 washes sterile distilled water. Seeds of each variety were then transferred to sterile 0.8% water agar and allowed to germinate in the dark for 3 days at 27˚C. Seedlings were transferred to Sigma tubes (150 x 25 mm) containing 20 ml of sterile fixed-nitrogen free RH medium supplemented with 0,20 or 400 µg of N per litre supplied as NH_4NO_3. The plants were grown in a growth cabinet at 26˚C with a 16 hr daylength and 314 µmol m-2S-1 of light. The seedlings were inoculated by either soaking in an inoculum before transfer to the Sigma tubes or, after 2 days of growth in the tubes, a 200 µl sample in TGYE medium of bacterial suspension (108-109 cells/ml) was introduced into the culture tubes. Uninoculated TGYM medium was used for the controls. For experiments with wheat (variety Wembley) similar procedures were employed.

Acetylene reduction assay. After 2, 4 or 8 weeks the Sigma culture tube closures were replaced by subaseals and 10% acetylene introduced into the culture tube. The plants were then grown under their normal growth conditions before a 0.5 ml air sample was removed and assayed for ethylene production by gas chromatography.

Fixation and embedding. Lateral roots (including STLRs) interacting with rhizobia were fixed and embedded for light and electron microscopy as previously described (6).

II – Results

1. Maize

Axenic seedlings of maize that had been inoculated with *Aeschynomene rhizobia* (ORS310) or *Parasponia rhizobia* (CP283) and grown in jars on RH medium lacking fixed nitrogen developed an extensive root system with numerous lateral roots. After three weeks, lateral roots (STLRs) were observed on the inoculated seedlings which were shorter and thicker than lateral roots formed on maize seedlings in the absence of rhizobia. STLRs, selected by their tetrazolium red staining, were taken at 3,4,5 and 8 weeks *(Table 1)*. When sections of STLRs were examined, after 3 weeks, using light microscopy, toluidine blue staining showed that rhizobia were invading the base of the emerging lateral root primordia and penetrating between cells at the base of the emerging lateral roots. After 4 weeks, rhizobia were detected penetrating extensively between the cells of the cortex of the STLRs towards the apex; by 5

and 8 weeks, cells of the cortex of the STLR had been invaded by rhizobia, with extensive accumulation of rhizobia within cortical cells. This suggests that cells of the cortex can be invaded by intercellular rhizobia without infection thread formation, as in Arachis, and that the invasion pathway of *Aeschynomene Rhizobium* strain ORS310 is by 'crack entry' at the point of emergence of lateral root primordia. This is somewhat similar to the 'crack entry' of rhizobia at the point of emergence of lateral root primordia in stem nodulation of *Sesbania* and *Aeschynomene* (7).

These studies on maize which had clearly indicated the initiation of lateral root nodule formation by 'crack entry' at the point of emergence of developing lateral roots led us to re-investigate the interaction of rhizobia with rice and wheat to see if such lateral root nodulation could be correlated with nitrogen fixation.

2. Rice

The acetylene reduction assay showed that both rice varieties, Lemont and IR42, developed nitrogen fixing activity following inoculation with ORS571. Rice plants (Lemont) produced ethylene from acetylene after inoculation with ORS571 and 2 weeks growth on media containing 0, 20 and 400 µgN/litre and also after 4 and 8 weeks growth. IR42 only produced ethylene after 2 weeks on medium containing 0 µgN/litre, and also after 4 weeks but not 8 weeks. No acetylene reduction activity was observed in the controls with either Lemont or IR42 seedlings (with no inoculation) cultured on the various media for 2, 4 or 8 weeks, or in culture tubes inoculated only with ORS571 and incubated for 2, 4 or 8 weeks in the growth room.

The detailed responses of rice seedlings (Lemont and IR42) interacting with ORS571 are shown in *Table 2A* (Lemont) and *Table 2B* (IR42). It was also established that there was a significant stimulation of lateral root formation following inoculation of both IR42 and Lemont with ORS571 under our test conditions.

Rice plants (IR42 and Lemont) inoculated with ORS571 that were active in nitrogen fixation (*Table 2A and 2B)* were fixed in 3% v/v glutaraldehyde in sodium phosphate buffer pH7 and embedded in Whites medium. Examination of the root system showed extensive lateral root formation, and some of the most active samples possessed a few short, thicker lateral roots (STLRs). When sections were examined rhizobia were seen invading at the base of emerging lateral roots and particularly into STLRs. Detailed analysis is continuing to further establish this correlation of initiation of lateral root nodule formation with nitrogen fixing activity.

3. Wheat

The acetylene reduction assay showed that wheat variety Wembley developed nitrogen fixing activity following inoculation with ORS571. Addition of up to 400 µg N/l was not inhibitory. No acetylene reduction activity was detected in the controls in which wheat seedlings (with no inoculation) were cultured on the various media for 2, 4 or 7 weeks, or in culture tubes inoculated only with ORS571 and incubated for 2, 4 or 7 weeks in the growth room. The detailed responses of wheat seedlings (Wembley) interacting with ORS571 are shown in *Table 3*. It was also established that there was a significant stimulation of lateral root formation following inoculation of these wheat seedlings with ORS571 under our test conditions.

Wheat (Wembley) plants inoculated with ORS571 that were active in nitrogen fixation *(Table 3)* were fixed and embedded as for rice. Examination of the root system showed extensive lateral root formation. The most active plants had numerous short, thicker lateral roots, and when sections of these were examined by light microscopy (staining with toluidine blue) rhizobia were seen to be invading these developing lateral root nodules both between and into the cells of the cortex surrounding the central vascular system. Detailed analysis is continuing to further establish this correlation of the initiation of lateral root nodule formation with nitrogen fixing activity.

Table 1. Detection using tetrazolium red of shorter, thicker lateral roots (STLRs) on maize seedlings interacting with Rhizobium strains ORS310 or CP283

Interaction with ORS310

Age of Plants (weeks)	**Number of Plants Analysed**	**Number of Plants with STLRs**	**% of Plants with STLRs**	**Average Number of STLRs/Plant**
3	132	8	6.1	3.1
4	40	3	7.5	1.6
5	108	6	5.5	3.6
8	251	12	4.8	4.0

Interaction with CP283

Age of Plants (weeks)	**Number of Plants Analysed**	**Number of Plants with STLRs**	**% of Plants with STLRs**	**Average Number of STLRs/Plant**
3	18	1	5.5	4.0
5	48	3	6.2	3.3
8	33	2	6.0	1.5

Table 2A. Rice variety Lemont : Inoculation with ORS571

Age of plants (weeks)	**µg N*/l**	**Number of plants**	**Range** (n moles ethylene/plant/24hr)	**Mean**** (n moles ethylene/plant/24hr)
2	0	171	0.4-265	28.3
	20	45	0.4-23.5	10.9
	400	20	2.6	2.6
4	0	42	4.3	4.3
	20	25	0	0
	400	18	0.5-0.6	0.6
8	0	20	6.6	6.6
	20	20	2.4-5.0	3.6
	400	20	2.5	2.5

Table 2B. Rice variety IR42 : Inoculation with ORS571

Age of plants (weeks)	**µg N*/l**	**Number of plants**	**Range** (n moles ethylene/plant/24hr)	**Mean**** (n moles ethylene/plant/24hr)
2	0	173	0.9-117	35
	20	45	0	0
	400	20	0	0
4	0	42	4.4	4.4
	20	25	0	0
	400	20	0	0
8	0	20	0	0
	20	20	0	0
	400	20	0	0

* Supplied as NH_4NO_3

** The mean amount of ethylene produced per plant is the mean for plants that showed acetylene reduction activity to produce ethylene.

Table 3. Wheat variety Wembley : Inoculation with ORS571

Age of plants (weeks)	µg N*/l	Number of plants	Range (n moles ethylene/plant/24hr)	Mean** (n moles ethylene/plant/24hr)
2 0	35	0.4-112	13.7	
20	34	0.3-129	13.3	
400	33	0.2-122	20.2	
4	0	33	0.7-9.5	3.1
20	33	0.4-7.5	2.6	
400	33	1.0-52.0	14.2	
8	0	30	0.7-4.6	3.6
20	30	3.0-3.8	3.4	
400	30	1.3-15.7	11.4	

* Supplied as NH_4NO_3

** The mean amount of ethylene produced per plant is the mean for plants that showed acetylene reduction activity to produce ethylene.

III – Discussion

It is now generally agreed that the introduction of symbiotic nitrogen fixation into the cereals would be one of the most significant contributions biotechnology could make to agriculture (8). The initial studies using maize interacting with *Parasponia* and *Aeschynomene rhizobia* indicated the likelihood that the initiation of lateral root nodule formation in cereals, and other non-legume crops, would establish a niche for symbiotic nitrogen fixation. The study of the interaction of *Azorhizobium caulinodans* (ORS571) with both rice and wheat has now shown significant levels of nitrogen fixation, as assessed using acetylene reduction assays, and a correlation of this nitrogen fixation with the invasion of emerging lateral roots by 'crack entry' and the initiation of lateral root nodule formation. ORS571 is known to form stem nodules on the tropical legume *(Sesbania rostrata)* that are of lateral root origin (9), and it is known that the nitrogenase activity of ORS571, unlike most rhizobia, is tolerant of low levels (2%) of oxygen (10). ORS310 which forms stem nodules on another tropical legume *(Aeschynomene indica)* is also known to have a nitrogenase that is tolerant to low levels of oxygen (10). These experiments on rice and wheat should therefore be repeated using *Rhizobium* ORS310 to determine whether significant levels of nitrogen fixation can also be obtained. Nitrogen fixation activity using 15N dilution assays and the use of Nif-mutants will also be advantageous. Experiments need also be undertaken to extend the length of time that rice and wheat seedlings can maintain nitrogen fixing ability in culture tubes, prior to the transfer of such seedlings to pots, by increasing the level of added fixed nitrogen. Attempts need to be undertaken to grow such inoculated rice and wheat plants to maturity in pots, and to analyse for nodule survival, nitrogen fixation and effects on crop growth and development. These experiments need to be further correlated with an examination of the efficiency of 'crack inoculation' through a study of the root system and the influence of plant growth conditions and inoculated rhizobia on lateral root formation. Nodulation factors (lipo-oligosaccharides) (11) that are secreted by ORS571 and ORS310, and known to stimulate nodule formation in their natural hosts, need to be evaluated for their effects on the interaction of these rhizobia with rice and wheat. These studies should provide a baseline of knowledge for the development of a commercial inoculation system. Since our present system does not need the addition of growth substances (such as 2,4-D) for the initiation of lateral root nodule formation, the development of such an inoculation system should largely parallel that already developed for the inoculation of legume crops with rhizobia.

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