

## Xyloglucanase Production by Rhizobial Species

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### Abstract

Xyloglucanase activity was evaluated in wild-type strains from different taxonomic groups of rhizobia that nodulate a specific range of legume hosts (*Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv. *viceae*, *R. leguminosarum* bv. *trifolii*, *R. tropici*, *Sinorhizobium* NGR, *Mesorhizobium ciceri*, *M. mediterraneum*). To detect endo- and exoxyloglucanase activities we used viscometric assay and quantitation of reducing groups released with a bicinchoninate reagent respectively. Endoxyloglucanase enzymes were detected in all the rhizobia tested both in the extracellular culture medium and in the intracellular fractions. Exoxyloglucanase activity was only found in the intracellular fraction of the rhizobia strains. These findings are consistent with the hypothesis of an involvement of rhizobial hydrolytic enzymes in the invasion of legume by *Rhizobium*.

Keywords: Endoxyloglucanase, exoxyloglucanase, hydrolytic enzymes, rhizobia

### 1. Introduction

Soil bacteria known as rhizobia have the capacity to induce the formation of nodules on the roots of their leguminous host plants. The precise mechanism

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whereby *Rhizobium* successfully infects temperate legumes remains unknown. Van Spronsen et al. (1994) proposed that polygalacturonase production by plant root cells, induced by homologous strains of *Rhizobium*, would result in an increase in plant cell wall softening at the infection site on the root hair; thus allowing the bacteria to penetrate the cell wall and initiate infection thread. Electron microscopic studies of the infection process have showed a localized degradation of the root hair wall, suggesting the involvement of cell wall hydrolytic enzymes in the penetration process (Callaham and Torrey, 1981; Higashi and Abe, 1980; Ridge and Rolfe, 1985; Turgeon and Bauer, 1985). The main point of divergence would be the possibility that the wall-degrading enzymes involved in the process are associated with the bacteria or locally induced in the plant by components of the bacteria.

In general, the activity of these rhizobial hydrolytic enzymes may be very low and localized at the site of infection in order to avoid destruction of the root hair (Dazzo and Hubbell, 1982). It may be that rhizobial cell wall degrading enzymes allow penetration without elicitation of a host plant defence. Although there is indirect evidence for the involvement of some hydrolytic enzymes in the infection process (Al-Mallah et al., 1987; Muñoz et al., 1998), little is known about the presence of these enzymes in *Rhizobium*. Although some studies failed to demonstrate the production of the hydrolytic enzymes by *Rhizobium* (Hunter and Elkan, 1975), several studies have detected pectinase (Hoflich and Kuhn, 1996; Hussain et al., 1995; Mateos et al., 1992), cellulase and hemicellulase (Akimova et al., 2000; Jimenez-Zurdo et al., 1996; Sprent, 1994; Struffi et al., 1998) enzyme activities from pure culture of rhizobia.

Xyloglucan is the major structural hemicellulose in the primary cell wall of plants. In addition to its structural role, xyloglucan can be hydrolysed by plant and microorganisms hydrolytic enzymes and the products used as a source of signalling molecules (Hayashi, 1989) and as a food reserve (Fry, 1989). Of the different hydrolytic enzymes, xyloglucanases are the less well known; however, they play an important role in plant cell wall degradation (Hoson et al., 1995). No studies on production of xyloglucanases by *Rhizobium* have been described.

The aim of this work was to study the presence of xyloglucan hydrolytic enzymes in several rhizobia strains supporting the hypothesis of an involvement of these rhizobial hydrolytic enzymes in the symbiosis.

## 2. Materials and Methods

The strains of rhizobia used in this study were *Sinorhizobium meliloti* GR4, *Rhizobium leguminosarum* bv. *viceae* Rlv 13, *R. leguminosarum* bv. *trifolii* 2152,

*R. tropici* CIAT 899, *Sinorhizobium* sp. NGR 234, *Mesorhizobium ciceri* LMG 14989 and *M. mediterraneum* USDA 3392 from the Estación Experimental del Zaidín (Granada).

Broth cultures were grown in 75 ml of B-INOS defined medium (Mateos et al., 1992) in 250 ml flasks shaken at 125 rev min<sup>-1</sup> at 28°C. Inocula were prepared by suspending cells from 5-day-old plate cultures into sterile B-INOS medium, centrifuging aseptically at 4,000 g for 15 min and resuspending in B-INOS medium to an initial population density of 10<sup>7</sup> cells ml<sup>-1</sup>. After 24 h, at 28°C, cells were pelleted by centrifugation, resuspended in 100 mM potassium phosphate-citric acid buffer (PCA; pH 5), sonicated in five cycles of 1 min bursts, and recentrifuged. This sonicated, cell-free extract and the original supernatant of extracellular culture fluid concentrated by ammonium sulphate were assayed for endo- and exoxyloglucanase activities. All enzyme samples were stored at 4°C. Similar experiment was carried out with *Rhizobium* grown in culture medium with xyloglucan as a sole carbon source.

To detect xyloglucanase activities we used two methods, viscometric assay and quantitation of reducing sugar products with a bicinchoninate reagent. Endoxyloglucanase activity was assayed by the viscosity method, using xyloglucan as substrate from nasturtium seed (*Tropaeolum majus* L.) extracted as described by McDougall and Fry (1989). The reduction in viscosity was determined at 0–30 min intervals. Approximately 0.5 ml of the reaction mixture was sucked into a 1 ml syringe and the time taken for the meniscus to flow from the 0.70 ml to 0.20 ml mark was recorded. The reaction mixture contained 1 ml of 0.5% substrate (0.5 mg/ml) in 50 mM citrate-phosphate buffer (pH 5) and 0.2 ml enzyme. Viscosity reduction was determined at 37°C. One unit of enzyme activity was expressed as total (U/ml) and as specific activity (U/mg prot). One enzyme unit means the reciprocal of time in h for 50% viscosity loss  $\times 10^3$  (Rejón-Palomares et al., 1996).

Xyloglucanase activity was also quantified by measuring the reducing groups released with a 2,2'-bicinchoninate reagent (BCA) which measure preferably exoxyloglucanase activity (Waffenschmidt and Jaenichke, 1987). Reaction mixtures at 40°C contained 400  $\mu$ l of 0.5% substrate in 50 mM citrate-phosphate buffer (pH 5), 25  $\mu$ l of the enzyme sample diluted to 400  $\mu$ l with H<sub>2</sub>O, and 800  $\mu$ l of 200 mM potassium phosphate-citric acid buffer (pH 5). Product formation was measured as described by Mateos et al. (1992). The enzyme activity was expressed as total and specific activity. One enzyme unit was defined as the amount (nmol) of reducing sugar released per h at 40°C and pH 5.

Appropriate controls were performed with heat-denatured extracts. Protein content of extracts were determined by the method of Bradford (1976) using a Biorad kit with BSA as the standard. The experiments were repeated three times, each data point is the average of replicate samples from one

representative experiment. The data were analysed by the one-way ANOVA followed by Duncan' multiple range test ( $P \leq 0.05$ ).

### 3. Results and Discussion

Xyloglucanase activities observed in *S. meliloti* GR4, *R. leguminosarum viceae* Rlv13, *R. leguminosarum trifolii* 2152, *R. tropici* CIAT 899, *S. sp* NGR 234, *M. ciceri* LMG 14989 and *M. mediterraneum* USDA 14989 are summarized in Table 1. This work demonstrated for the first time the presence of endo- and exoxyloglucanase enzymes in rhizobia. Production of other hydrolytic enzymes by *Rhizobium* has been studied (Akimova et al., 2000; Hoflich and Kuhn, 1996; Hussain et al., 1995; Jimenez-Zurdo et al., 1996; Mateos et al., 1992; Sprent, 1994; Struffi et al., 1998). Some of them, such as cellulase, are commonly produced by diverse *Rhizobium* strains; but other hydrolytic enzymes such as polygalacturonases seem to be produced only by *R. leguminosarum trifolii* strains (Jimenez-Zurdo et al., 1996; Mateos et al., 1992). Our results show that all rhizobia strains tested were able to produce xyloglucanase activities.

Xyloglucanase activity in the extracellular and intracellular extracts of each strain was evaluated by a viscometric assay, specific for measuring endoxyloglucanase activity (Rapp and Beermann, 1991). With this method endoxyloglucanase activity either in the extracellular and intracellular extracts of all strains of rhizobia were observed. Our results show that total endoxyloglucanase activity inside the *Rhizobium* cells was similar to that of cell exudates, indicating that all rhizobia strains liberate great quantity of endoxyloglucanase enzymes to the growth medium.

Although a small quantity of proteins was observed in the growth medium of rhizobia, the great permeability of rhizobia to endoxyloglucanase proteins may explain the higher specific activity of this enzyme in the extracellular fraction of rhizobia cells. The production of extracellular endoxyloglucanase enzyme by rhizobial suggests that these enzymes can play an important role in the processes of penetration of rhizobia in the host plant cell. The production of cell wall-degrading enzymes by *Rhizobium* is stimulated by the presence of compatible host tissue polysaccharides or polysaccharide-containing moieties (Iannetta et al., 1997). The fact that higher levels of endoxyloglucanase activities were detected in *Rhizobium* culture medium supplemented with xyloglucan than in the B-INOS media (data not shown) reinforce this idea.

The quantitative BCA-reducing group assay, more specific for measuring exoxyloglucanase activity (Jimenez-Zurdo et al., 1996), showed different amount of xyloglucanase specific activities among the strains used (Table 1).

The data presented in this study show that the intracellular fraction of the strains *S. meliloti* GR4, *R. leguminosarum viceae* Rlv 13, *R. leguminosarum*

Table 1. Extracellular and intracellular xyloglucanase activities of different *Rhizobium* species.

<i>Rhizobium</i> test strain	Exo-xyloglucanase		Intra-xyloglucanase		Exo-xyloglucanase	
	Extracellular		Intracellular		Intracellular	
	Total (U/ml)	Specific (U/mg prt)	Total (U/ml)	Specific (U/mg prt)	Total (U/ml)	Specific (U/mg prt)
<i>S. meliloti</i> GR4	30.1 b	4,430 d	44.3 b	1,600 c	0.40 a	21.70 b
<i>R. leguminosarum viceae</i> Riv 13	28.1 b	2,418 d	25.3 b	387 c	0.20 a	3.10 a
<i>R. leguminosarum trifolii</i> 2152	28.6 b	2,799 d	39.5 b	850 c	0.015 a	0.27 a
<i>R. tropici</i> CIAT 899	50.5 b	2,203 c	58.6 b	5,012 d	0.004 a	0.03 a
<i>Sinorhizobium</i> . sp NGR 234	24.6 b	1,154 c	34.4 b	1,012 c	0.005 a	0.04 a
<i>M. cicer</i> LMG 14989	37.4 b	37,350 e	52.5 b	8,750 c	0.008 a	0.18 a
<i>M. mediterraneum</i> USDA 3392	26.6 b	2,633 d	29.9 b	320 c	0.008 a	0.06 a

Each value is the mean of three replicates derived from equivalent number of cells. Within each *Rhizobium* species row values followed by the same letter are not significantly different according to Duncan's multiple range test ( $P \leq 0.05$ ). There was no extracellular exo-xyloglucanase activity determined by the reducing sugar assay in any of the different *Rhizobium* species tested.

*trifolii* 2152, *R. tropici* CIAT 899, *S. sp* NGR 234, *M. ciceri* LMG 14989 and *M. mediterraneum* USDA 3392 produced xyloglucanolytic enzymes with exo hydrolytic activity. However, no exoxyloglucanase activity in the concentrated extracellular fraction of each strain was observed, indicating that these enzymes are cell-bound.

Several authors reported that CMC-cellulase and polygalacturonase activities from different rhizobia species are cell-bound (Jimenez-Zurdo et al., 1996; Mateos et al., 1992), however, Iannetta et al. (1997) found that cellulase activity of *R. leguminosarum viceae* Rlv 13 was restricted mainly to the culture supernatants. In summary, our study indicate that endoxyloglucanase activity is produced by a diversity of species of root-nodule bacteria either in extracellular and intracellular extracts however the exoxyloglucanase activity is only found in the intracellular fraction of rhizobia.

The low production of xyloglucanolytic enzymes that cleave glycosidic bonds in plant cell wall polymers has been show to be a general phenomenon among various species of *Rhizobium*. This low activity of this enzyme strongly suggests a possible role in the root invasion process by this microorganism. Purification and further characterization of this enzyme should make it feasible to investigate the possible function of these polysaccharide-degrading enzymes in the establishment of the symbiotic interactions.

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