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Résumé de l'article

Les rhizobiums arctiques isolés à partir d'*Astragalus* et d'*Oxytropis spp*. sont adaptés au froid et offrent un potentiel pour l'amélioration de la fixation symbiotique d'azote à basse température. Ainsi ils ont été utilisés dans des études de nodulation et pour des essais au champ avec une légumineuse fourragère tempérée, le sainfoin (*Onobrychis viciifolia*). Un point majeur d'inquiétude pour l'inoculation des légumineuses est l'effet potentiellement toxique des tannins produits par les graines et les racines sur le rhizobium. Nous avons étudié les effets des exsudats des graines de sainfoin sur la croissance de 47 souches de rhizobium arctique et de 2 souches de rhizobium du sainfoin. La croissance de toutes les souches testées sur la gélose au mannitol et à l'extrait de levures a été inhibée à différents degrés par les exsudats des graines de sainfoin. Nous n'avons pas observé de corrélation entre la zone d'inhibition et l'efficacité symbiotique de chaque souche sur le sainfoin. Cependant, l'inhibition de la croissance a été complètement renversée par l'addition de fer sous forme de Fe-EDTA dans le milieu gélose. En milieu liquide sans l'ajout de fer, la croissance des souches arctiques après 4 jours était inhibée à 94%. Avec l'addition de 100 µM de Fe-EDTA, l'inhibition n'était que de 5 % et avec 100 µM de citrate de fer, elle était de 76 %. Ces résultats soulignent l'importance d'évaluer les effets toxiques sous des conditions rhizosphériques normales.

Growth inhibition of rhizobia isolated from arctic legumes (*Astragalus* and *Oxytropis* spp.) and sainfoin (*Onobrychis viciifolia*) by sainfoin seed diffusates

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Cold-adapted arctic rhizobia (from *Astragalus* and *Oxytropis* species) were used in nodulation studies and field inoculation trials with a temperate forage legume, sainfoin (*Onobrychis viciifolia*), since they have the potential to improve symbiotic nitrogen fixation at low temperatures. A major concern in legume inoculation is the toxicity of seed and root tannins to rhizobia. We studied the effects of sainfoin seed diffusates on the growth of 47 arctic and 2 sainfoin strains of rhizobia. Growth of all tested strains on yeast-extract mannitol agar plate was inhibited to various degrees by sainfoin seed diffusates. There was no correlation between the zone of inhibition and the symbiotic effectiveness of each strain on sainfoin. However, growth inhibition was totally reversed by the addition of iron as Fe-EDTA in the agar medium. In liquid medium without iron, growth inhibition of an arctic strain was 94 % after 4-day growth. With the addition of 100 μM Fe-EDTA, inhibition was only 5 %, and with 100 μM Fe-citrate, 76 %. These results underline the importance of evaluating the toxicity effect under normal rhizospheric conditions.

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Les rhizobiums arctiques isolés à partir d'*Astragalus* et d'*Oxytropis* spp. sont adaptés au froid et offrent un potentiel pour l'amélioration de la fixation symbiotique d'azote à basse température. Ainsi ils ont été utilisés dans des études de nodulation et pour des essais au champ avec une légumineuse fourragère tempérée, le sainfoin (*Onobrychis viciifolia*). Un point majeur d'inquiétude pour l'inoculation des légumineuses est l'effet potentiellement toxique des tannins produits par les graines et les racines sur le rhizobium. Nous avons étudié les effets des exsudats des graines de sainfoin sur la croissance de 47 souches de rhizobium arctique et de 2 souches de rhizobium du sainfoin. La croissance de toutes les souches testées sur la gélose au mannitol et à l'extrait de levures a été inhibée à différents degrés par les exsudats des graines de sainfoin. Nous n'avons pas observé de corrélation entre la zone d'inhibition et l'efficacité symbiotique de chaque souche sur le sainfoin. Cependant, l'inhibition de la croissance a été complètement renversée par l'addition de fer sous forme de Fe-EDTA dans le milieu gélosé. En milieu liquide sans l'ajout de fer, la croissance des souches arctiques après 4 jours était inhibée à 94 %. Avec l'addition de 100 μM de Fe-EDTA, l'inhibition n'était que de 5 % et avec 100 μM de citrate de fer, elle était de 76 %. Ces résultats soulignent l'importance d'évaluer les effets toxiques sous des conditions rhizosphériques normales.

Introduction

The temperate perennial forage legumes sainfoin (*Onobrychis viciifolia* Scop.) can be effectively nodulated by rhizobia isolated from arctic legumes (*Astragalus* and *Oxytropis* spp.) (Prévost *et al.* 1987b). These cold-adapted rhizobia have the potential to improve symbiotic nitrogen fixation of sainfoin at low temperatures (Prévost *et al.* 1987a). Sainfoin has potential in western Canada and the United States as pasture crop or hay because it is resistant to the major alfalfa pests, and is non-bloating for cattle (Cash 1982; Hanna *et al.* 1980). However, sainfoin seeds and roots produce phenolic compounds identified as condensed tannins that inhibit the growth of rhizobia (Ames-Gottfred 1981), as it has been frequently reported for several legume species (Masterson 1965; Materon and Weaver 1984; Peters and Alexander 1966; Ranga Rao *et al.* 1973; Young and Paterson 1980). The toxicity of seeds is a major ecological concern in *Rhizobium*-legume inoculation since survival of rhizobia in the rhizosphere is a prerequisite to nodulation and nitrogen fixation. As arctic rhizobia have the potential to be used as sainfoin inoculants, we evaluated the growth inhibition of 47 arctic strains by sainfoin seed diffusates in yeast extract mannitol medium.

Since observation of growth inhibition of rhizobia by clover seeds and tannic acid can be eliminated by the addition of iron to the

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medium (El-Zamik and Wright 1987), we also studied the effects of iron on the inhibition of arctic rhizobia by sainfoin seed diffusates.

Materials and methods

***Rhizobium* strains.** Arctic rhizobia used in this study were previously described (Prévost *et al.* 1987c). Two temperate strains of rhizobia homologous to sainfoin were also used: strain SM-2, isolated from sainfoin cultivated in Saskatchewan and strain 116A15, used in commercial inoculant (Nitragin Co., Milwaukee, Wisconsin). Cultures were maintained on yeast extract mannitol agar medium (YMA) (Vincent 1970).

Seed diffusates. Dehulled seeds of sainfoin cv. Melrose and cv. Nova were sterilized under a gas mixture of 12% ethylene oxide (Oxyfume 12: 12% ethylene oxide, 88% fluorocarbon$_{12}$) for 72 h. This treatment did not affect seed viability as demonstrated by the unaltered rate of germination. One hundred grams of sterilized seeds were soaked in 200 mL of sterile distilled water for 24 h at 5°C under aeration. Seed sterility was demonstrated by the fact that no bacterial or fungal growth was detected on YMA plates inoculated with seed diffusates. Finally, seed diffusates (100 mL final volume) were filtered successively through a Whatman no. 1 paper and a Millipore filter (0.45 μm).

Toxicity tests. All strains were tested on two media (YMA and YMA supplemented with 400 μM Fe-EDTA) against diffusates of the two sainfoin seed cultivars. For each strain, two Petri dishes of each medium were surface inoculated using a cotton swab drenched in a fresh standardized inoculum (O.D. = 0.7 at 630 nm) containing 10^8 cells/mL. Two sterile 10 mm filter paper disks were moistened with 100 μL of seed diffusates and placed on the agar surface of each plate. After 3 days of incubation for temperate strains (mean generation time 2.5-3.0 h) and after 7 days for arctic strains (mean generation time 4.0-8.0 h) at 25°C, the zone of inhibition was measured from the edge of the disk to that of the bacterial growth. In liquid media, two different sources of iron (Fe-EDTA and Fe-citrate) were also tested at concentrations of 100 μM and 400 μM Fe in 100 mL of yeast extract mannitol broth (YMB) containing different amounts (0.2, 0.4, 0.8, and 1.6 mL) of seed diffusates of sainfoin cv. Nova. Three flasks of each treatment were inoculated each with 10^6 cells (20 μg protein) of the arctic strain N_{28} and incubated at 25°C for 5 days on a rotary shaker (125 r/min). Cells from 1 mL culture were taken daily, washed twice in saline buffer (NaCl, 0.85%), resuspended in 1 mL 1N NaOH and heated at 90°C for 10 min prior to protein determination by the modified Folin procedure (Hanson and Phillips 1981).

Statistical analysis. All data were submitted to analysis of variance and means were compared using the least significant difference test (LSD).

Results and discussion

Toxicity effects and symbiotic effectiveness. Strains of arctic rhizobia showed great variation in their sensitivity to sainfoin seed diffusates when tested with imbibed filter paper disks on YMA medium (Table 1). The arctic strain N_{28} was the most sensitive to both sainfoin cultivar diffusates with an inhibition zone greater than 22 mm. The most resistant arctic strains to 'Melrose' seed diffusates, N_{35} and N_{36}, were also resistant to 'Nova' seed diffusates. However, one strain, N_{45}, was about three times more sensitive to 'Nova' than to 'Melrose' diffusates and, inversely, some strains (N_2, N_4 and N_{15}) were more sensitive to 'Melrose' than to 'Nova' diffusates. These results are similar to those obtained with other species of legumes and rhizobia, where the degree of inhibition varies according to the legume seed and to the bacterial strain (Dadarwal and Sen 1973; Masterson 1962). Except for strain N_2, which was the least sensitive arctic strain to 'Nova' seed diffusates, inhibition zones of temperate strains SM-2 and 116A15 were the smallest for both cultivars. The large gum production on surface-inoculated agar plates and the rapid growth rate of sainfoin strains SM-2 and 116A15 in comparison to arctic strains could have masked a greater toxicity effect. However, it has been suggested that extracellular gum production may provide

Table 1. Toxicity effects of seed diffusates of two sainfoin cultivars on the growth of arctic and sainfoin rhizobia on YMA plate

Rhizobial strain	Symbiotic effectiveness§	Zone of inhibition of sainfoin cultivar (mm)†	
		'Melrose'	'Nova'
Arctic			
N_1	I	5.62	7.00
N_2	I	6.00	2.00
N_3	I	5.37	4.25
N_4	I	8.62	2.87
N_5	I	4.75	6.00
N_6	I	19.50	19.50
N_7	I	12.00	7.00
N_8	H	10.25	14.00
N_9	H	10.50	13.25
N_{10}	H	12.00	13.00
N_{11}	I	5.12	8.62
N_{12}	I	6.37	4.87
N_{13}	I	6.50	2.75
N_{14}	I	8.50	6.00
N_{15}	M	7.75	3.00
N_{16}	I	5.75	4.75
N_{17}	M	6.75	5.75
N_{18}	I	8.75	5.75
N_{19}	I	9.62	13.00
N_{20}	I	8.25	3.00
N_{21}	I	6.00	4.50
N_{22}	I	7.62	4.25
N_{23}	I	8.37	3.62
N_{24}	I	5.75	5.12
N_{25}	I	7.12	12.87
N_{26}	I	6.12	5.12
N_{27}	I	4.75	5.25
N_{28}	I	22.75	26.25
N_{29}	I	4.75	2.25
N_{30}	I	8.25	4.50
N_{31}	H	11.25	7.75
N_{32}	M	7.88	6.00
N_{33}	M	5.88	5.62
N_{34}	I	5.62	5.12
N_{35}	I	3.75	3.00
N_{36}	I	4.00	4.12
N_{37}	I	6.25	3.62
N_{38}	I	6.75	4.25
N_{39}	H	9.00	11.75
N_{40}	H	8.50	10.25
N_{41}	I	6.37	12.25
N_{42}	I	5.12	11.12
N_{43}	I	6.00	11.50
N_{44}	I	6.25	8.50
N_{45}	I	6.37	19.37
N_{46}	I	6.12	5.50
N_{47}	M	5.75	6.75
Temperate			
SM-2	—	2.00	2.00
116A15	—	2.00	2.00
LSD (0.05)		0.85	1.30

§ Symbiotic effectiveness (SE) arbitrarily rated as ineffective (I) if SE < 50%, moderately effective (M) if SE = 50 to 70% or highly effective (H) if SE > 70%, determined by comparison of the shoot dry weight of sainfoin (cv. Melrose) inoculated with the test arctic strain with that obtained with the temperate strains SM-2 and 116A15 (Prévost *et al.* 1987b).

† Each value is the mean of four replicates.

some protection to rhizobia from toxic seed diffusates (Dadarwal and Sen 1973).

In liquid culture the toxicity response of the most sensitive arctic strain N_{28} was compared to that of the weakly sensitive sainfoin strain SM-2. Relative growth of strain SM-2 (Fig. 1b) showed two phases in growth inhibition. One day after inoculation, growth was totally inhibited by 1.6 and 0.8 mL seed diffusates, and relative growth yield with 0.4 and 0.2 mL was 43 % and 61 % of growth without seed diffusates. At day 2, a growth increase observed with every amount of seed diffusates used, suggested a delaying effect of seed diffusates on growth initiation. From day 2 to day 5, a net inhibition was observed with 0.8 and 1.6 mL while lesser amounts showed a weaker inhibitory effect. Growth of the arctic strain N_{28} showed a different inhibition pattern (Fig. 1a) where inhibitory effect was not detected at day 1, but gradually appeared with time. At day 4, growth with 0.8 mL seed diffusates was only 6 % of its growth without seed diffusates while it was 61 % for the sainfoin strain SM-2. Overall, growth inhibition of arctic strain N_{28} was stronger than that of sainfoin strain SM-2, as it was also observed on surface-inoculated agar. However, the two phases of growth inhibition of strain SM-2 observed at day 1 and at day 4-5 reveal a greater toxicity effect than that observed on agar plate. Ames-Gottfred (1981) has shown that toxicity response of sainfoin rhizobia differs from strain to strain as do quantities of tannins in sainfoin roots and seeds, although no direct correlation could be found between them.

Sensitivity of arctic rhizobia to sainfoin seed diffusates was not related to their symbiotic effectiveness on sainfoin at the first cutting (Table 1), since there was no correlation (r = 0.0392) between the size of inhibition zone and the shoot dry mass of sainfoin inoculated with arctic strains. However, the highly effective temperate strains SM-2 and 116A15 were less sensitive to seed diffusates than arctic strains. Toxicity of phenolic substances towards natural microbial pests has been considered an important function of these plant metabolites, but the real effect on rhizobia and on nitrogen fixation remains unknown. In fact, tannins are found in sainfoin nodules as well as in other plant tissues, leaves, roots and seeds (Ames-Gottfred 1981; Jones and Mangan 1977). Thus, there may exist a mechanism whereby some strains of rhizobia can grow and develop an efficient symbiosis in nodules of sainfoin in spite of the presence of tannins. However, the toxicity may have an effect on survival of

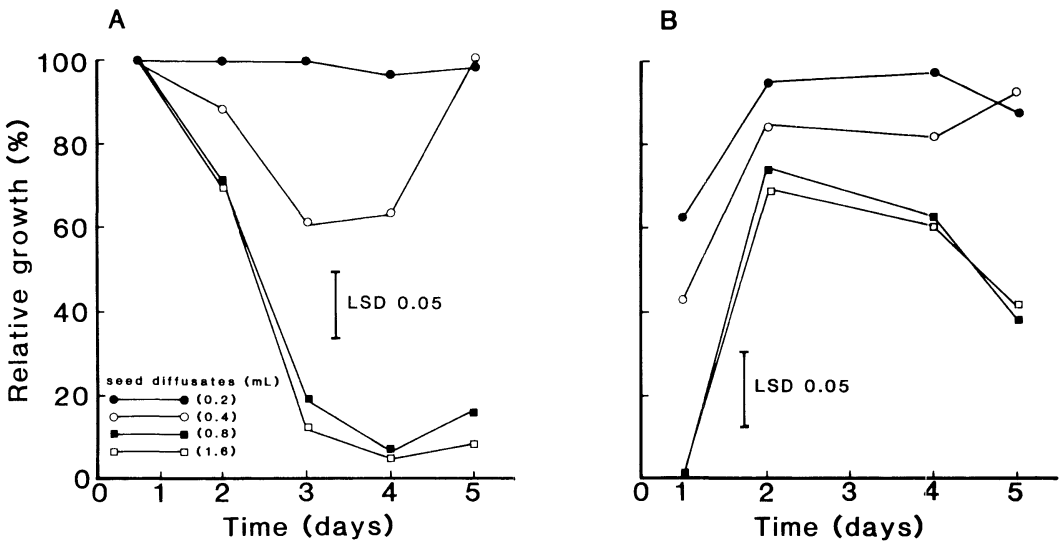

Figure 1. Relative growth of the arctic strain N_{28} (**A**) and the temperate strain SM-2 (**B**) in the presence of different amounts of sainfoin seed diffusates. Relative growth (%) = yield (μg/mL protein) obtained with a known amount of seed diffusates divided by the yield obtained without seed diffusates (X 100).

rhizobia on the seed or in the rhizosphere, as reported elsewhere (Hale and Mathers 1976; Materon and Weaver 1984).

Effect of iron on toxicity. No inhibition zones were observed on solid YMA plates containing 400 μM Fe-EDTA. Similar results were observed with clover rhizobia tested against clover seeds and tannic acid on agar media where growth inhibition was alleviated with the presence of 100 μM Fe-EDTA in the media (El-Zamik and Wright 1987). In order to characterize the growth response of rhizobia to seed diffusates in the presence of iron, the growth yield of the arctic strain N_{28} as measured as mg of protein produced per mL of culture was followed with time in liquid medium (Fig. 2). In the standard YMB, increasing amounts of seed diffusates resulted in a strong growth inhibition. The toxic effect of seed diffusates on rhizobial cells appeared to be bacteriostatic since the growth yield obtained with the largest amount of seed diffusates (1.6 mL) was almost stable from day 1 to day 5 with a range of 15 to 30 μg/mL protein (Fig. 2). This yield is higher than the initial inoculum level indicating some cell division. Viability of rhizobial cells in all treatments was demonstrated by the presence of rhizobial colonies on YMA plates after streaking. However, the addition of 100 μM or 400 μM Fe-EDTA to the medium reversed the observed inhibition, as measured by the final growth yield and growth was enhanced as observed by a shorter lag phase after inoculation. The concentration of 100 μM Fe-EDTA was the most efficient to reverse growth inhibition since at day 4, growth with high amounts (0.8 and 1.6 mL) of seed diffusates was similar to that obtained with no seed diffusates, while it was still very low with 400 μM Fe-EDTA.

The reversion of growth inhibition by seed diffusates was also studied with ferric citrate (Table 2). As with Fe-EDTA, Fe-citrate was more efficient at 100 μM than at 400 μM to reduce the inhibition of the arctic strain N_{28} by seed diffusates. However, 100 μM Fe-EDTA works better than 100 μM Fe-citrate since growth with 1.6 mL seed diffusates was inhibited by only 5% in comparison to the 76% inhibition with 100 μM Fe-citrate. Without iron in the media, growth was inhibited by 94%. As tannins are chelating agents, the reversion of inhibition may be due to the fact that ferric ions bind to tannins diffused from the seeds to form complexes which are not toxic to rhizobia. The differences in efficiency of both sources of iron may be related to their different affinity to bind to tannins. The concentration of 100 μM may correspond to an optimal number of Fe-tannins complexes needed to reverse toxicity effects.

The sensitivity of rhizobia to sainfoin seed diffusates, detected only in yeast extract mannitol medium, did not seem to affect their symbiotic effectiveness on sainfoin. Possibly, in the evaluation of strain effectiveness (Prévost *et al.* 1987b), the bacteriostatic effect of diffusates did not interfere

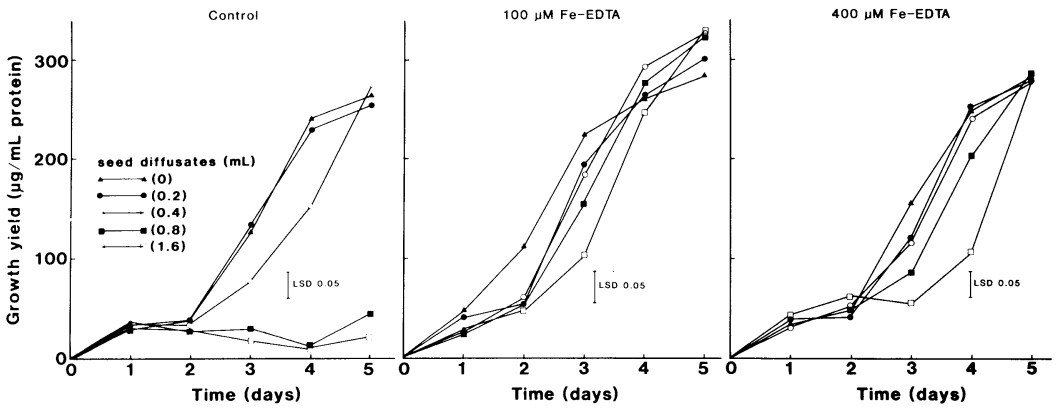

Figure 2. Effects of different concentrations of iron as Fe-EDTA on the growth yield of the arctic strain N_{28} in the presence of different amounts of sainfoin seed diffusates.

Table 2. Effect of different sources of ferric ions on the growth inhibition of arctic strain N_{28} by seed diffusates of sainfoin, after 4-day growth in yeast extract mannitol medium

		Seed diffusates (mL)									
		0		0.2		0.4		0.8		1.6	
Source of iron	Concentration of ferric ions (μM)	Growth yield (μg/mL)	Inhibition (%)	Growth yield (μg/mL)	Inhibition (%)	Growth yield (μg/mL)	Inhibition (%)	Growth yield (μg/mL)	Inhibition (%)	Growth yield (μg/mL)	Inhibition (%)
Control	0	242§	0†	231	5	143	41	16	93	15	94
Fe-EDTA	100	260	0	268	0	294	0	278	0	248	5
	400	250	0	256	0	239	4	206	17	215	16
Fe-Citrate	100	296	0	298	0	302	0	163	45	71	76
	400	289	0	273	6	209	18	158	45	51	82

§ Growth yield as determined by the protein content (μg/mL). Each result is the mean of three replicates.

† Percentage of inhibition = $(1 - \frac{\text{growth yield with a specific amount of seed diffusates}}{\text{growth yield without seed diffusates}}) \times 100$

with the inoculation at a level to prevent nodulation. The toxicity effect could also have been reversed by metal ions present in the nutrient solution, since it has been reported that the addition of different metal salts alleviates the inhibition of rhizobial growth by seed diffusates (Masterson 1965).

Furthermore, it is difficult to establish the ecological significance of this sensitivity because growth inhibition is strong only in the presence of large quantities of seed diffusates (100 μL on paper disks and 1.6 mL seed diffusates are equivalent to the effect of 6 and 100 sainfoin seeds, respectively) which may not be representative of the possible dilution of tannins under rhizosphere conditions. In soils, phenolic compounds are exuded from plant roots and produced from humic acid degradation; their ability to chelate with metal ions such as Fe^{+3} (Schnitzer 1978) may reduce the toxicity effect on rhizobia. However, this phenomenon may be significant in soils poor in available iron where both the plant and the bacteria do have specific requirements for their optimum growth. Further studies are required to understand the mechanism whereby the presence of iron reverses the inhibitory effect of seed diffusates.

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