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# Symbiotic effectiveness of indigenous arctic rhizobia on a temperate forage legume: Sainfoin (*Onobrychis viciifolia*)*

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**Abstract**
Sainfoin (*Onobrychis viciifolia*), a temperate perennial forage legume, can be nodulated by rhizobia isolated from 3 arctic legume species: *Astragalus alpinus, Oxytropis maydelliana* and *Oxytropis arctobia*. Arctic rhizobia, which are adapted to growth at low temperatures, may be useful in improving symbiotic nitrogen fixation during cold phases of the growing season, if they are effective on a temperate legume. In this study, we report on the symbiotic effectiveness of arctic rhizobia on sainfoin, as appraised by the total shoot dry matter yield obtained from 2 harvests. Under N-free conditions, 5 arctic strains at the first harvest and 8 at the second harvest were as effective as temperate standard strains. In the presence of $30\,mg\,l^{-1}$ $NO_3$-N, 7 arctic strains gave significantly higher yields than temperate strains at the second harvest. These results indicate that effective arctic rhizobia have a potential for use as inoculants on sainfoin.

## Introduction

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial forage legume that has good potential in western Canada and the United States, and has several advantages overa alfalfa (*Medicago sativa* L.). In fact, sainfoin is resistant to the major alfalfa pests, non-bloating, relatively drought tolerant and winterhardy, it can also be used for pasture or hay and its forage is similar in quality to that of alfalfa (Cash, 1982; Hanna *et al.*, 1980).

Sainfoin has a level of nitrogenase activity per unit plant weight similar to other forage legumes (Hume *et al.*, 1985; Krall and Delaney, 1982). However, in the early stage of growth, one of the major problems seems to be the inefficiency of the $N_2$-fixing system system to meet the plant's nitrogen requirements (Burton and Curley, 1968). Relative low growth rate and low leaf area ratio probably reduce the availability of energy for symbiotic nitrogen fixation, resulting in a poor production of plant nitrogen (Hume *et al.*, 1985). The presence of low levels of mineral N to inoculated plants increases the forage yield (Burton and Curley, 1968; Hill, 1980). However, on plants initially grown in symbiotic conditions only, the addition of mineral N substitutes for, rather than supplements, nitrogen fixation (Hume and Withers, 1985). Therefore, in studying the effectiveness of rhizobia on sainfoin, it might be necessary to consider the effects of mineral nitrogen which favours the plant's establishment but replaces nitrogen fixation.

The most effective rhizobia reported on sainfoin originate from this plant, but the latter can also be effectively nodulated by some strains of rhizobia isolated from the legumes *Coronilla* spp, *Hedysarum* spp, and *Petalostemon* spp, (Ames-Gottfred, 1981; Burton and Curley, 1968; Cash, 1982). Recently, we found that rhizobia isolated from

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three arctic legume species (*Astragalus alpinus* L., *Oxytropis maydelliana* Trautv and *O. arctobia* Bunge) were able to nodulate sainfoin (Prévost *et al.*, 1987). These arctic rhizobia also showed an adaptation to growth at 5°C and hence their potential may be useful in improving symbiotic nitrogen fixation mainly by enhancing early nodulation and nitrogenase activity at low soil temperature. The present study seeks to evaluate the symbiotic effectiveness of arctic rhizobia on the temperate legume sainfoin under N-free conditions and in the presence of low levels of nitrate-N.

## Materials and methods

### *Origin and maintenance of rhizobia*

Arctic rhizobia used in this study were previously described (Prévost *et al.*, 1987). They were isolated from *A. alpinus* (21 strains): $N_1$ to $N_5$, $N_{18}$, $N_{19}$, $N_{21}$, $N_{24}$, $N_{27}$, $N_{29}$ to $N_{31}$, $N_{34}$ to $N_{36}$, $N_{39}$, $N_{42}$ and $N_{44}$ to $N_{46}$, from *Oxytropis maydelliana* (19 strains): $N_6$ to $N_{13}$, $N_{15}$, $N_{17}$, $N_{20}$, $N_{22}$, $N_{26}$, $N_{28}$, $N_{32}$, $N_{37}$, $N_{40}$, $N_{41}$, and $N_{43}$, and from *Oxytropis arctobia* (8 strains): $N_{14}$, $N_{16}$, $N_{23}$, $N_{25}$, $N_{33}$, $N_{38}$, $N_{47}$ and $N_{48}$. The two standard temperate strains used as controls were the effective strain $SM_2$, isolated from sainfoin cultivated in Saskatchewan and strain 116A15, used in a commercial inoculant (Nitragin Co., Milwaukee, Wi) and reported as very effective on sainfoin cv Melrose (Ames-Gottfred, 1981). Rhizobia were maintained on yeast-extract mannitol (YEM) (Vincent, 1970) agar slants. The inocula were prepared by growing the rhizobial strains in 250 ml Erlenmeyer flasks containing 100 ml of yeast extract mannitol broth and incubated at 25°C for 6 days on a rotary shaker (160 rev. $min^{-1}$).

### *Symbiotic effectiveness under N-free conditions*

The symbiotic effectivenesss of the 48 arctic rhizobia was assessed with dehulled seeds of sainfoin cv Melrose, the only cultivar on the market in Canada (Hanna *et al.*, 1980). Uniformly sized seeds were surface sterilized in $HgCl_2$ (Vincent, 1970) and germinated on sterile agar (1%) for 36 h in the dark. Five uniform germinated seeds were planted in Riviera pots (Manufacture provençale de matières plastiques de Marseille, France) sterilized with 0.5% Oakite solution (Sanitizer no. 1, Oakite Products of Canada, Bramalea, Ontario) and containing 2.3 l of an autoclaved mixture of 50% (v/v) vermiculite in sand. The pot reservoirs were filled with a N-free Hoagland's nutrient solution (Bordeleau *et al.*, 1977). Every 2 weeks, pots were flushed and refilled with fresh nutrient solution. Distilled water was added to the pots when required. Plants were inoculated 7 and 35 days after planting by adding to each pot 100 ml of the nutrient solution containing approximately 18° *Rhizobium* cells $ml^{-1}$. At day 21, pots were thinned to two uniform plants per pot. From day 21 to 51, a small amount of N (30 mg $l^{-1}$ N as $KNO_3$) was added to the nutrient solution to facilitate plant establishment. Plants were grown under a 16-h light period (350 $\mu$E.$m^{-2}$. $s^{-1}$) at 20°C and 8-h darkness at 15°C. The experimental design was a randomized complete block with three replicates. The treatments included the 48 arctic strains, the two standard strains $SM_2$ and 116A15, one uninoculated treatment without nitrogen and one uninoculated treatment receiving 140 mg $l^{-1}$ N as $NH_4NO_3$. Plants were harvested at the beginning of anthesis: 80 days (first harvest) and 110 days (second harvest) after sowing. Plants were dried at 80°C for 24 h in a forced-air oven, weighed, ground and analysed for total N by a micro- Kjeldahl method (Ward and Johnston, 1962).

All data, except uninoculated treatments, were submitted to statistical analysis and means were compared by using the least significant differences (LSD) (Steel and Torrie, 1960).

The symbiotic effectiveness of the arctic strains was determined by comparison with the two standard strains $SM_2$ and 116A15 according to the formula (Gibson, 1980):

$$\text{S.E., \%} = \frac{\text{S.D.W. inoculated test strain}}{\text{S.D.W. inoculated standard}} \times 100$$

where S.E. is the symbiotic effectiveness of a strain and S.D.W. is the shoot dry weight of the plants inoculated with the strain under test or the average of the two standard strains $SM_2$ and 116A15. The effectiveness of the strains was arbitrarily rated as high (H), moderate (M) or ineffective (I) according to the following scale: H: S.E. > 70%; M: S.E. = 50–70%; I: S.E. < 50%.

*Symbiotic effectiveness of some strains in the presence of a low N level*

The 12 arctic strains used in this assay were selected on the basis of their symbiotic effectiveness at the first harvest under N-free conditions: $N_8$, $N_{10}$, $N_{31}$ and $N_{40}$ (highly effective); $N_{15}$, $N_{17}$, $N_{33}$ and $N_{47}$ (moderately effective) and $N_{11}$, $N_{28}$, $N_{30}$ and $N_{44}$ (ineffective). The assay was performed as described earlier with the following modifications. Plants were inoculated 15 days after planting, and pots were thinned at day 21 to one plant per pot. For the duration of the assay, the nutrient solution was supplemented with $30\,mg\,l^{-1}$ N (as $KNO_3$). A composite commercial inoculant (Nitragin) was also tested by adding enough peat to the nutrient solution to give approximately $10^8$ rhizobial cells $ml^{-1}$. Two uninoculated treatments were included, one receiving $30\,mg\,l^{-1}$ N as $KNO_3$ and the other $140\,mg\,l^{-1}$ N as $NH_4NO_3$. The experimental design was a randomized complete block with eight replicates. Plants were harvested at the beginning of anthesis: 50 days (first harvest) and 80 days (second harvest) after sowing. Determination of plant dry weight, N content, statistical analysis and appraisal of symbiotic effectiveness were performed as described in the first assay. As well, plant nitrogen derived from symbiotic fixation was estimated according to the formula:

$$\%\ \text{N.D.F.} = \frac{\text{N-test strain} - \text{N-uninoculated}}{\text{N-test strain}} \times 100$$

where % N.D.F. is the approximate percentage of nitrogen derived from the atmosphere through symbiotic fixation; N-test strain is the total nitrogen content of the plants inoculated with the strain under test in the presence of $30\,mg\,l^{-1}$ $NO_3$-N; N-uninoculated is the total nitrogen content of the uninoculated plants grown in the presence of $30\,mg\,l^{-1}$ of $NO_3$-N. The % N.D.F. was not determined in the assay under N-free conditions because uninoculated plants were not able to survive in the absence of a continuous source of low level nitrogen.

## Results and discussion

*Symbiotic effectiveness of arctic rhizobia under N-free conditions*

Among the 48 arctic rhizobia tested, 17 did not sustain regrowth of sainfoin after the first harvest and their dry matter yield was low and insufficient to perform any analysis. Hence, only the symbiotic effectiveness of the remaining 31 strains is reported in Table 1. At the first harvest, the highest shoot dry matter yield (S.D.W.) was obtained with the arctic strain $N_8$ but was not statistically different from yields obtained with the arctic strains $N_9$, $N_{10}$, $N_{31}$ and $N_{39}$ or the standard strains $SM_2$ and 116A15. The total N-content obtained with the three arctic strains $N_8$, $N_9$, $N_{10}$ and the temperate strain 116A15 were also not statistically different from the highest value obtained with the strain $SM_2$. When compared to the two standard strains, six arctic strains showed a high symbiotic effectiveness (S.E.).

The shoot dry matter weight of sainfoin receiving $140\,mg\,l^{-1}$ N as $NH_4NO_3$ was higher than yields obtained with plants depending only on symbiotic nitrogen fixation. This could be attributed to the relatively low growth rate of sainfoin dependent on symbiotic $N_2$ fixation, and to the greater root than top development of these plants during the early stage of growth (Hume and Withers, 1985). Similar results were previously observed with a population of *R. meliloti* on alfalfa (Bordeleau *et al.*, 1977).

At the second harvest, the highest shoot dry matter yield observed with strain $N_{10}$ was not significantly different from the yields obtained with eight arctic strains or the two standard strains (Table 1). Also, 11 arctic strains and the two temperate strains $SM_2$ and 116A15 were not statistically different in their total N-content. When compared to the two standard strains, a total of 17 arctic strains showed a high symbiotic effectiveness. None of the effective strains decreased in symbiotic effectiveness at the second cutting. On the contrary, the number of effective strains increased. In fact, among the 20 strains rated ineffective at the first cutting, seven became highly effective and one was moderately effective at the second cutting. This observation might be an indication of a good reinfection and nodulation of well developed roots after the first cutting. Furthermore, the six arctic

*Table 1.* Growth response of sainfoin to inoculation and symbiotic effectiveness of arctic rhizobia under N-free conditions

| Strains | Harvest | | | | | |
|---|---|---|---|---|---|---|
| | 1 | | | 2 | | |
| | S.D.W.[a] ($g\,pot^{-1}$) | S.E.[a] (%) | Total N ($mg\,pot^{-1}$) | S.D.W. ($g\,pot^{-1}$) | S.E. (%) | Total N ($mg\,pot^{-1}$) |
| *Arctic* | | | | | | |
| $N_1$ | 0.96 | 18.7 I[b] | 19 | 0.69 | 18.0 I | 13 |
| $N_7$ | 1.13 | 22.0 I | 22 | 1.28 | 33.5 I | 23 |
| $N_8$ | 5.32a[c] | 103.6 H | 123a | 4.47a | 116.9 H | 114a |
| $N_9$ | 4.66a | 90.7 H | 140a | 4.19a | 109.5 H | 100a |
| $N_{10}$ | 4.21a | 82.0 H | 118a | 4.98a | 130.2 H | 125a |
| $N_{11}$ | 1.89 | 36.8 I | 39 | 1.44 | 37.9 I | 27 |
| $N_{15}$ | 3.16 | 61.5 M | 68 | 2.54 | 66.4 M | 53 |
| $N_{16}$ | 1.07 | 20.8 I | 18 | 1.06 | 27.7 I | – |
| $N_{17}$ | 3.31 | 64.5 M | 75 | 2.95 | 77.1 H | 48 |
| $N_{20}$ | 0.48 | 9.4 I | 8 | 0.19 | 5.0 I | – |
| $N_{21}$ | 2.35 | 45.8 I | 59 | 3.28 | 85.8 H | 87a |
| $N_{24}$ | 2.46 | 47.9 I | 52 | 3.64a | 95.4 H | 84a |
| $N_{26}$ | 2.18 | 42.4 I | 38 | 3.15 | 82.4 H | 81a |
| $N_{27}$ | 2.46 | 47.9 I | 55 | 4.55a | 119.0 H | 108a |
| $N_{28}$ | 1.98 | 38.6 I | 49 | 2.42 | 63.5 M | 61 |
| $N_{30}$ | 1.24 | 24.1 I | 20 | 1.18 | 31.1 I | – |
| $N_{31}$ | 4.39a | 85.5 H | 100 | 3.14 | 82.1 H | 56 |
| $N_{32}$ | 3.46 | 67.4 M | 70 | 4.37a | 114.2 H | 92a |
| $N_{33}$ | 3.17 | 61.7 M | 94 | 4.84a | 126.5 H | 119a |
| $N_{34}$ | 2.36 | 46.0 I | 50 | 2.93 | 76.9 H | 77 |
| $N_{35}$ | 0.76 | 14.8 I | 8 | 0.43 | 11.2 I | – |
| $N_{38}$ | 0.83 | 16.2 I | 14 | 0.76 | 19.9 I | – |
| $N_{39}$ | 4.30a | 83.7 H | 95 | 3.36a | 88.1 H | 72 |
| $N_{40}$ | 3.70 | 72.0 H | 84 | 3.58a | 93.9 H | 98a |
| $N_{41}$ | 2.15 | 41.9 I | 59 | 3.08 | 80.5 H | 78 |
| $N_{42}$ | 1.37 | 26.7 I | – | 1.10 | 29.0 I | – |
| $N_{43}$ | 0.93 | 18.1 I | 16 | 0.94 | 24.6 I | 21 |
| $N_{44}$ | 1.20 | 23.4 I | 20 | 1.36 | 35.6 I | 32 |
| $N_{45}$ | 1.59 | 31.0 I | 31 | 1.69 | 44.2 I | 40 |
| $N_{46}$ | 2.15 | 41.9 I | 54 | 3.09 | 80.8 H | 75 |
| $N_{47}$ | 3.16 | 61.5M | 84 | 3.14 | 82.1 H | 81a |
| *Temperate* | | | | | | |
| $SM_2$ | 5.09a | | 145a | 3.32a | | 104a |
| 116A15 | 5.18a | | 144a | 4.33a | | 134a |
| LSD (0.05) | 1.54 | | 40 | 1.69 | | 54 |
| F (strains) | 6.86** | | 8.80** | 5.60** | | 3.17** |
| *Uninoculated controls* | | | | | | |
| N-free | 0.07 | | – | – | | – |
| 140 mg/l-N (as $NH_4NO_3$) | 7.31 | | 113 | 3.83 | | 75 |

[a] S.D.W., Shoot dry weight; S.E., Symbiotic effectiveness.
[b] H = high, S.E. > 70%; M = moderate, S.E. = 50–70%; I = ineffective, S.E. < 50%.
[c] means in the same column followed by the same letter are not significantly different from the highest shoot dry weight according to the LSD test at $p = .05$.
** Significant at $p < .01$.
– Not determined.

strains with symbiotic effectiveness greater than 100% and the two standard strains allowed shoot dry matter yields similar or slightly higher than those obtained with plants grown with 140 mg $l^{-1}$ $NH_4NO_3$, indicating that effective strains can meet the nitrogen requirements of sainfoin plants once they are established. The shoot dry matter weight of sainfoin plants was significantly related to their total-N content at the first (r = 0.95**) and the second (r = 0.92**) harvest.

This assay indicates that in nodulated sainfoin, plant growth is dependent of the early establishment of an effective symbiotic system. It has been reported that sainfoin rhizobia are not effective in supplying adequate amounts of nitrogen under N-free conditions (Burton and Curley, 1968), and we observed symptoms of N deficiency in all inoculated treatments in the early stage of growth but they disappeared with time in plants nodulated by effective strains.

Under N-free conditions, several arctic rhizobia were rated highly effective, regardless of their host plant origin. Thus, they might be useful as inoculants on sainfoin. In general for forage legumes, it has been suggested that the symbiotic effectiveness of rhizobia is better estimated in the presence of low levels of $NO_3$-N, similar to that found in soils (Heichel and Vance, 1983). However, the $N_2$-fixing system of sainfoin is very sensitive to the presence of low levels of nitrate (Hume and Withers, 1985). Therefore, we further studied the symbiotic effectiveness of 12 arctic rhizobia in the continuous presence of 30 mg $l^{-1}$ $NO_3$-N. The strains were selected on the basis of their symbiotic

*Table 2.* Growth response of sainfoin inoculated with 12 arctic strains in presence of low level $NO_3$-N

| Strains | Harvest | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | | | | 2 | | | |
| | S.D.W.[a] (g $pot^{-1}$) | S.E.[a] (%) | Total-N (mg $pot^{-1}$) | N.D.F.[a] (%) | S.D.W. (g $pot^{-1}$) | S.E. (%) | Total-N (mg $pot^{-1}$) | N.D.F. (%) |
| *Arctic* | | | | | | | | |
| $N_8$ | 2.38 | 107.9 | 43 | 53.5 | 3.98 | 125.0 | 91 | 83.5 |
| $N_{10}$ | 2.50 | 113.4 | 41 | 51.2 | 4.48a | 140.7 | 98 | 84.7 |
| $N_{11}$ | 2.53 | 114.7 | 35 | 42.8 | 4.33a | 135.9 | 70 | 78.6 |
| $N_{15}$ | 2.01 | 91.2 | 28 | 28.6 | 2.63 | 82.6 | 47 | 68.1 |
| $N_{17}$ | 2.63 | 119.3 | 43 | 53.5 | 5.08a | 159.5 | 114a | 86.8 |
| $N_{28}$ | 2.50 | 113.4 | 50a[b] | 60.0 | 5.05a | 158.5 | 111a | 86.5 |
| $N_{30}$ | 2.26 | 102.5 | 33 | 39.4 | 3.34 | 104.9 | 66 | 77.3 |
| $N_{31}$ | 2.59 | 117.5 | 46a | 56.5 | 4.57a | 143.5 | 100 | 85.0 |
| $N_{33}$ | 2.10 | 95.2 | 29 | 31.0 | 3.36 | 105.5 | 81 | 81.5 |
| $N_{40}$ | 3.00 | 136.0 | 54a | 62.9 | 5.56a | 177.7 | 147a | 89.8 |
| $N_{44}$ | 2.61 | 118.4 | 38 | 47.4 | 4.87a | 152.9 | 97 | 84.5 |
| $N_{47}$ | 2.29 | 103.9 | 30 | 33.3 | 3.96 | 124.3 | 92 | 83.7 |
| *Temperate* | | | | | | | | |
| Commercial inoculant | 2.56 | 111.6 | 63a | 68.2 | 3.51 | 110.2 | 81 | 81.5 |
| $SM_2$ | 2.20 | | 58a | 65.5 | 3.10 | | 66 | 78.5 |
| 116A15 | 2.21 | | 49a | 59.2 | 3.27 | | 87 | 82.8 |
| LSD (0.05) | – | – | 19 | – | 1.36 | – | 42 | – |
| F (strains) | 1.26 N.S. | | 2.45** | | 3.26** | | 2.49** | – |
| *Uninoculated control* | | | | | | | | |
| 30 mg $l^{-1}$-N-$NO_3$ | 2.07 | – | 20 | – | 1.48 | – | 15 | – |
| 140 mg $l^{-1}$ N (as $NH_4NO_3$) | 7.73 | – | 106 | – | 5.02 | – | 81 | – |

[a] S.D.W., Shoot dry weight; S.E., Symbiotic effectiveness; N.D.F., Approximative amount of nitrogen derived from symbiotic fixation.
[b] Means in the same column followed by the same letter are not significantly different from the highest shoot dry weight yield or the total-N content according to the LSD test at $p < .05$.
N.S. Not significant.
** Significant at $p < 0.01$.

effectiveness at the first cutting under N-free conditions.

*Symbiotic effectiveness of arctic rhizobia in the presence of low levels of $NO_3$-N*

In the presence of 30 mg $l^{-1}$ $NO_3$-N, the rhizobial strains had no significant effect on sainfoin shoot dry matter yields, at first harvest (Table 2) and the yields appeared to be similar to those obtained with the uninoculated control. This might suggest that sainfoin plants are using preferably nitrate in the early stage of growth. It has been also reported (Hume and Withers, 1985) that the application of 35 mg $l^{-1}$ $NO_3$-N on nodulated sainfoin plants replaces, rather than supplements, nitrogen fixation. However, the fact that all inoculated plants had a total N content higher than that of the uninoculated control, and that the approximate percentage of nitrogen derived from symbiotic fixation (% NDF) varied from 33.3% to 68.2%, indicates that an active $N_2$ fixing system depending of the strains is probably present (Table 2). It is known, in fact that strains of rhizobia differ in their effectiveness in the presence of mineral nitrogen (Gibson, 1980).

At the second harvest, seven arctic strains gave yields significantly higher than those obtained with the standard strains and the commercial inoculant. The highest N content was obtained with the three arctic strains $N_{17}$, $N_{28}$ and $N_{40}$. Plant total-N appears to be derived mainly from symbiotic nitrogen fixation as suggested by the higher percentage values of NDF (77.3% to 86.5%) compared to those observed at the first harvest. The former are in agreement with results previously reported (Hume *et al.*, 1985) indicating that sainfoin has an overall $N_2$-fixing activity per unit plant weight similar to those of other forage legumes. As observed under N-free conditions, the shoot dry matter weight of sainfoin grown in the presence of 30 mg $l^{-1}$ $NO_3$-N was significantly correlated to its total-N content at the first (r = 0.65***) and the second (r = 0.86***) harvest. However, as reported earlier (Hume and Withers, 1985), relationships between plant N and plant dry matter appeared to be closer in plant dependent only on nitrogen fixation than in nodulated plants growing with 30 mg $l^{-1}$ $NO_3$-N as it is shown by the higher coefficient of correlation found under N-free conditions.

Whether in the presence of nitrate or not, many arctic strains showed a high symbiotic effectiveness on sainfoin. Among the strains tested in both assays, the three arctic strains $N_{10}$, $N_{31}$ and $N_{40}$ demonstrated a good potential on sainfoin because of their high symbiotic effectiveness at first and second harvest in a N-free environment and their high shoot dry matter yields, total N content and % NDF, at the two harvests in the presence of 30 mg $l^{-1}$ $NO_3$N. These strains, adapted to growth at low temperature, can be used in further studies on nodulation, nitrogenase activity, and field inoculation trials of sainfoin.

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