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Effects of low temperatures on nitrogenase activity in sainfoin (*Onobrychis viciifolia*) nodulated by arctic rhizobia *

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1. SUMMARY

The temperate forage legume sainfoin (*Onobrychis viciifolia*) is readily nodulated by rhizobia isolated from arctic legumes (*Astragalus* and *Oxytropis* species). We have investigated the effects of low temperatures on nitrogenase activity in sainfoin nodulated by arctic and temperate (homologous) rhizobia. At low temperatures, nitrogenase activity of arctic rhizobia measured either with detached nodules or with whole plants, was higher than that of temperate rhizobia. At 5°C and 10°C, nitrogenase activity values of arctic rhizobia represented 12% and 33% of those measured at 20°C, while lower values of 3.7% and 22.4% were observed with temperate rhizobia. This cold adaptation was also reflected on bacterial growth where, at 5°C and 10°C, arctic rhizobia showed a shorter doubling time and synthesized more protein than temperate rhizobia.

2. INTRODUCTION

Symbiotic nitrogen fixation is dependent on the host plant genotype, the *Rhizobium* strain, and the interaction of these symbionts with the environment. Low temperatures have been shown to adversely affect both nodule formation and the rate of N₂ fixation by nodulated roots [1,2]. In temperate climates, the early growth of legumes occurs when soil temperatures are far below the optimum range for the growth of rhizobia and for nitrogen fixation by the symbiotic association [3]. Consequently, the enhancement of the nitrogen-fixing efficiency of agricultural legumes at low temperatures would be helpful in sustaining plant growth during cold phases of the growing season. The strain of *Rhizobium* plays an important role in determining the efficiency of nitrogen fixation at low temperatures [4,5]. Strains of *R. trifolii* isolated from subarctic regions of Scandinavia showed better growth, earlier nodulation and higher nitrogenase activity with clover at 10°C than those isolated from southern regions, while no significant differences were observed between isolates at 20°C [3]. In experiments simulating cold and warm climates of Finland, northern strains of *R. trifolii* showed better nitrogenase

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activity than southern strains in the cold climate [6].

The symbiotic nitrogenase activity of *Rhizobium* strains at different temperatures has been correlated to the responses of strains to temperature in pure culture [3,6,7]. Recently, we reported that rhizobia indigenous to the Canadian high arctic were able to grow at 5°C and readily nodulated the temperate forage legume sainfoin (*Onobrychis viciifolia* (Scop.)) [8]. Therefore, it is interesting to evaluate the potential of arctic rhizobia to increase the nitrogen fixing ability of sainfoin at low temperatures. In the present work, we report on the short-term effects of temperature on nitrogenase activity of juvenile sainfoin (established at optimum temperatures for the growth of the plants) nodulated with strains of *Rhizobium* isolated from arctic and temperate regions. The effect of temperature on growth of these bacteria was also investigated.

3. MATERIALS AND METHODS

3.1. Origin and maintenance of rhizobia

Arctic rhizobia used in this study were previously described [8]. Strains N₁₁ and N₂₈ were isolated from *Oxytropis maydelliana* and strain N₃₁ from *Astragalus alpinus*. The two temperate strains used for comparison were strain SM-2 isolated from *O. viciifolia* cv Melrose cultivated in Saskatchewan and strain 116A15 used in a commercial inoculant (Nitragin Co., Milwaukee, WI). Rhizobia were maintained on yeast-extract mannitol (YEM) [9] agar slants.

3.2. Growth characteristics at different temperatures

A fresh culture of each strain was prepared by growing cells in 50-ml Erlenmeyer flasks containing 10 ml of YEM broth and incubated at 25°C on a rotary shaker (125 rev · min⁻¹). At the mid-exponential phase, duplicate 1 ml of the culture were subcultured each into 10 ml of fresh medium and incubated for 4–40 days according to the temperature. Periodically (every 4 h at 30°C, 8 h at 25°C, 12 h at 20°C, 20 h at 15°C, 2 days at 10°C and 5 days at 5°C) bacterial cells from 1 ml aliquot were collected and heated in 1 ml 1 N

NaOH for 10 min at 90°C and the protein content was measured by the Folin reaction [10]. The mean generation time was calculated during the exponential phase.

3.3. Plant inoculation and growth

Inoculum was prepared by growing rhizobia in 250-ml Erlenmeyer flasks containing 100 ml of YEM broth and incubated at 25°C for 6 days on a rotary shaker (160 rev · min⁻¹). Uniformly sized seeds of sainfoin cv Melrose were surface sterilized with 0.2% HgCl₂ [9]. Two seeds were planted in each 25 × 160 mm Cone-Tainer tube (Ray Leach Co., Comby, Or.) sterilized with 0.5% Oakite solution (Sanitizer No. 1, Oakite Products of Canada, Bramalea, Ont.) and containing an autoclaved mixture of 50% (v/v) vermiculite in sand. Plants were grown under a 16-h light period (350 μEm⁻² · s⁻¹) at 20°C and 8 h darkness at 15°C. At day 7, seedlings were thinned to one plant per tube. At day 14 and 21, each seedling was inoculated with approximately 10⁸ rhizobial cells. Plants were fed weekly with 10 ml of Hoagland's nutrient solution [8] containing 10 mg N as KNO₃ per liter. Distilled water (10 ml) was applied twice a week. Plants were watered the day before digging for the acetylene reduction assay.

3.4. Acetylene reduction assay (ARA) conditions

Plant material was always collected at the same time of day, 5 h after the beginning of the photoperiod. Individual whole plants (5 replicates) or 300 mg (fresh weight) of detached nodules selected from 3 different plants (3 replicates) were placed in 18 × 150 mm glass tubes equilibrated in a controlled water bath at the selected assay temperature. After 5 min equilibration, the tubes were sealed with Suba-Seal (William Freeman & Co. Barnsley) stoppers. Before the injection of 10% (v/v) acetylene, the same volume of air was removed. The tubes were incubated under mild agitation. Gas samples (0.2 ml) were taken after 30 min, 1 h, and 2 h incubation at 25°, 30°, and 35°C; after 1 h, 2 h and 4 h at 20°C; and after 2 h, 4 h, and 8 h at 5°, 10°, and 15°C. The samples were analysed for ethylene content on a Perkin-Elmer gas chromatograph equipped with a Porapak-R-column and flame ionisation detector

according to the described procedure [11]. The dry weight of shoots and nodules was determined after drying at 80 °C for 48 h in a forced air oven. Nitrogenase activity was calculated for the linear phase of the reaction and was expressed in $\mu\text{mol C}_2\text{H}_4\text{h}^{-1} \cdot \text{g}^{-1}$ nodule dry weight. Statistical analysis was performed for each temperature by comparison of means according to Duncan's multiple range test [12].

4. RESULTS

4.1. Effects of temperature on bacterial growth

Growth characteristics from 5 °C to 30 °C of the arctic strain N_{31} and the temperate strain SM-2 are illustrated in Table 1. Optimum growth temperatures were 25 °C for the strain N_{31} and 30 °C for the strain SM-2. The growth of the 2 strains decreased gradually with decreasing temperature. However, at 10 °C the arctic strain showed a shorter doubling time than the temperate strain, and at 5 °C the growth of strain N_{31} was very slow but strain SM-2 was unable to grow. At 15 °C and 20 °C, little differences were observed, and at 25 °C and 30 °C, the temperate strain grew faster than the arctic strain. Growth yield was estimated by the amount of protein obtained at the stationary phase of the growth curve at each temperature. For the arctic strain, protein synthesis was at its maximum ($\sim 440 \mu\text{g}/\text{ml}$) from 5 °C to 25 °C, and very poor ($\sim 100 \mu\text{g}/\text{ml}$) at 30 °C. The growth yield of the tem-

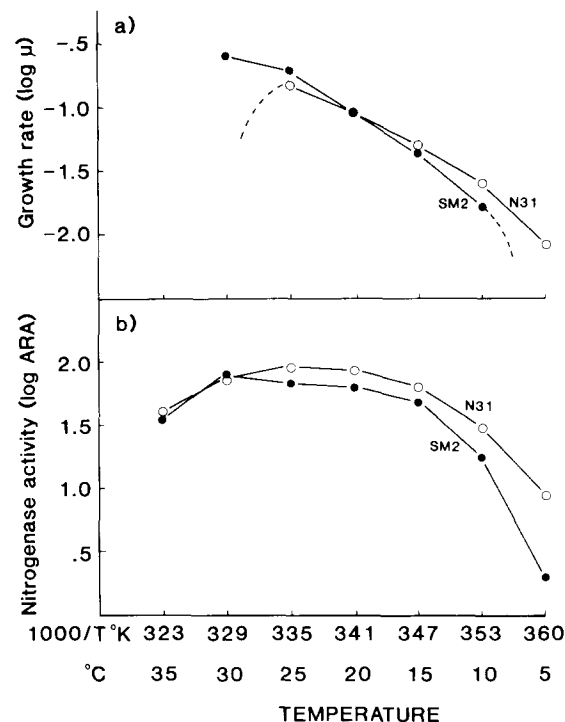


Fig. 1. Arrhenius plots of bacterial growth (a) and nitrogenase activity (b) of the arctic strain N_{31} and temperate strain SM-2. (a) μ = number of generations/h, calculated according to the formula $\mu = 0.693/g$, where g is the doubling time reported in Table 1. Dotted lines are projected values since no growth was observed at 30 °C for the strain N_{31} and at 5 °C for the strain SM-2. (b) ARA: acetylene reduction activity in $\mu\text{mol C}_2\text{H}_4\text{h}^{-1} \cdot \text{g}^{-1}$ nodule dry weight, with detached nodules.

Table 1

Growth characteristics of arctic and temperate rhizobial strains at different temperatures

| Strains | Temperature | | | | | |
|-----------------------------------|-------------|-------|-------|-------|-------|-----------------|
| | 5 °C | 10 °C | 15 °C | 20 °C | 25 °C | 30 °C |
| Arctic N_{31} | | | | | | |
| Doubling time ^a | 82.5 | 27.3 | 14.0 | 9.0 | 4.5 | ND ^c |
| Growth yield ^b | 446 | 438 | 428 | 428 | 432 | 104 |
| Temperate SM-2 | | | | | | |
| Doubling time | ND | 42.8 | 15.6 | 8.0 | 3.6 | 2.8 |
| Growth yield | 12 | 320 | 370 | 446 | 468 | 464 |

^a Expressed in hours.

^b Expressed in μg protein/ml protein at the stationary phase.

^c Not determined, the maximum growth yield was too low.

perate strain was at its maximum from 20°C to 30°C, and decreased gradually to almost no growth at 5°C. The Arrhenius plot of the bacterial growth (Fig. 1a) illustrates that both strains responded linearly to temperatures from 25°C to 15°C, with a steeper slope for the temperate strain.

4.2. Effects of rhizobial strains on nitrogenase activity at different temperatures

After 70 days of growth, plants infected with the 2 arctic strains N_{31} and N_{28} and the 2 temperate strains SM-2 and 116A15 displayed similar shoot dry weight (Table 2). The arctic strain N_{11}

showed the lowest shoot dry weight and was the least effective. However, nodule mass of plants inoculated with the temperate strain SM-2 was higher than that obtained with plants nodulated with any of the 3 arctic strains. Specific nitrogenase activity, expressed per g nodule dry weight is reported in Table 3. With whole plants (undetached nodules), at 5°C and 10°C, the arctic strains N_{31} and N_{28} showed higher nitrogenase activity than the temperate strains. At 15°C and 20°C, the activity of the strain N_{11} was the lowest.

Nitrogenase activity was also measured on detached nodules in order to eliminate the effect of temperature on plant metabolic processes (Table 3). In general, results with detached nodules show the same tendency as those observed with whole plants. However, at 10°C, the arctic strain N_{31} showed the highest activity and the temperate strain SM-2 was similar to the arctic strain N_{28} . At 15°C, both arctic strains N_{31} and N_{28} were the highest.

Nitrogenase activity at temperatures higher than 20°C was assayed with detached sainfoin nodules developed with the temperate strain SM-2 and the arctic strain N_{31} . The Arrhenius plot of the acetylene reduction assay (Fig. 1b) does not show a linear relationship as observed with the bacterial growth (Fig. 1a). In the low-temperature range, from 15°C to 5°C, nitrogenase activity of the temperate strain declined more rapidly than that

Table 2

Nodules and shoot dry weights of 70-day-old sainfoin plants nodulated with arctic and temperate strains of rhizobia

| Bacterial strain | Shoot dry weight (mg/plant) | Nodule dry weight (mg/plant) |
|------------------|-----------------------------|------------------------------|
| Arctic | | |
| N_{31} | 143.6 a ^a | 35.8 bc |
| N_{28} | 104.6 ab | 39.8 bc |
| N_{11} | 72.8 b | 24.0 c |
| Temperate | | |
| SM-2 | 147.6 a | 63.8 a |
| 116A15 | 133.6 ab | 53.4 ab |

^a Means within a column followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Table 3

Nitrogenase activity at low temperatures of whole plant (undetached nodules) and detached nodules of sainfoin inoculated with arctic and temperate strains ($\mu\text{mol C}_2\text{H}_4\text{h}^{-1}\cdot\text{g}^{-1}$ nodule dry weight)

| Strains | Temperature | | | | | | | |
|-----------|----------------------|------------------|--------------|------------------|--------------|------------------|--------------|------------------|
| | 5°C | | 10°C | | 15°C | | 20°C | |
| | Whole plants | Detached nodules | Whole plants | Detached nodules | Whole plants | Detached nodules | Whole plants | Detached nodules |
| Arctic | | | | | | | | |
| N_{31} | 12.11 a ^a | 8.66 a | 35.23 a | 29.61 a | 61.03 a | 63.50 ab | 130.00 a | 86.03 a |
| N_{28} | 5.87 b | 9.49 a | 37.51 a | 17.21 bc | 56.09 a | 74.08 a | 91.45 b | 83.88 a |
| N_{11} | 3.78 c | 6.06 b | 18.16 b | 19.90 b | 35.70 b | 14.34 d | 51.42 c | 44.65 b |
| Temperate | | | | | | | | |
| SM-2 | 3.45 cd | 2.12 c | 21.36 b | 18.84 bc | 52.14 a | 49.77 c | 81.21 b | 63.47 ab |
| 116A15 | 1.94 d | 2.56 c | 14.82 b | 15.41 c | 45.97 ab | 55.75 bc | 80.32 b | 62.75 ab |

^a Means within a column followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

of the arctic strain. In the high-temperature range, from 20°C to 35°C, the nitrogenase activity of the temperate strain increased slightly up to 30°C (66.80 and 84.76 $\mu\text{mol C}_2\text{H}_4\text{h}^{-1}\cdot\text{g}^{-1}$ at 25° and 30°C, respectively). The activity of the arctic strain was at its maximum at 25°C (92.66 $\mu\text{mol C}_2\text{H}_4\text{h}^{-1}\cdot\text{g}^{-1}$), and started to decrease at 30°C (79.09 $\mu\text{mol C}_2\text{H}_4\text{h}^{-1}\cdot\text{g}^{-1}$). These results were statistically different between the two strains at 25°C, but not at 30°C and 35°C where the nitrogenase activity declined drastically (39.41 and 35.25 $\mu\text{mol C}_2\text{H}_4\text{h}^{-1}\cdot\text{g}^{-1}$ for the strains N₃₁ and SM-2, respectively).

5. DISCUSSION

Free-living arctic rhizobia are much better adapted to growth at 5°C when compared to the temperate rhizobia tested. At 5°C, the 3 arctic strains synthesized 10 times more protein (results not shown) than temperate strains. The fact that the arctic strain N₃₁ grew faster than the temperate strain SM-2 at 5°C and 10°C, and that little differences were observed at 15°C, 20°C and 25°C demonstrated clearly an adaptation that resulted in an ability to synthesize protein at low temperatures. By using the Arrhenius plot (Fig. 1a), growth responses between these two strains were shown to be similar to those found between psychrophilic and mesophilic bacteria [13]. In fact, differences can be observed in the slope of the linear part of the plots and in the temperature range of growth where, at low temperatures, the arctic strain grows better than the temperate strain. Although the nitrogenase activity of *Rhizobium* strains have been correlated to the responses of strains to high [7] and low [3] temperatures in pure culture, the growth rate of a strain at a low temperature alone did not predict its symbiotic ability in a cold climate [6].

In our study, evidence of an adaptation of the ability of the 3 arctic rhizobia (including the less effective strain N₁₁) to fix nitrogen at low temperatures is demonstrated by the fact that they expressed, at 5°C and 10°C, an average of 12% and 33% of their activity at 20°C, while the temperate strains showed an average of only 3.7%

and 22.4% (calculated for detached nodules).

The Q₁₀ value (the number of times by which an enzymatic activity is increased by a 10°C rise in temperature) of the arctic strain N₃₁ was 3.3 between 5°C and 15°C. This indicates that the nitrogenase system formed by this strain is affected by temperature like simple enzymatic reactions which show a Q₁₀ of about 2. Similar values have also been reported for the nitrogenase activity of detached nodules of alfalfa [15]. However, the high Q₁₀ value (23.2) of the strain SM-2 between 5°C and 15°C, considering its low activity at 5°C, may indicate either that the low temperatures have affected other factors influencing nitrogenase activity, as reported before [5,14] or that the nitrogenase itself is more sensitive to low temperatures. Between 15°C and 25°C, the Q₁₀ values were lower, but similar between the 2 strains (1.5 for N₃₁ and 1.3 for SM-2).

The Arrhenius plot of nitrogenase activity of sainfoin showed discontinuities similar to those observed on several plant species [2]. The discontinuity appeared near 15°C with nodules formed by the 2 strains N₃₁ and SM-2, but the nitrogenase activity of SM-2 declined more rapidly when the temperature fell from 15°C to 5°C. At high temperatures, discontinuity occurred at 30°C with strain SM-2 and near 25°C with the arctic strain N₃₁.

It has been suggested that indigenous rhizobia isolated from wild type legumes may have useful properties not found in *Rhizobium* strains from cultivated crops [16]. We suggest that some of the metabolic activities involved in the symbiotic nitrogen fixation process were affected by arctic rhizobia. It could be that arctic rhizobia have a higher permease activity and/or a higher affinity for some substrates in the nodule than the temperate strains. In fact, psychrophilic bacteria have the ability to transport sugar efficiently at low temperatures [17]. It is also possible that arctic rhizobia are using specific photosynthates as energy source for the nitrogenase reaction. These photosynthates might be present either with arctic or temperate strains, or synthesized only when arctic rhizobia are microsymbionts in the sainfoin symbiosis.

The observed effect of temperature may also be

reflected in the photosynthate cost and the relative efficiency of the nitrogen fixing system which may differ between arctic and temperate strains. In fact, in many symbioses, temperature affects also the ratio of H_2 involved in the enzymatic system of nitrogenase, and the electron allocation to this enzyme [4,5]. Differential responses to temperature may also be a consequence of the structure of the functioning nodule [4] or an intrinsic property of the enzyme.

The present work shows that arctic rhizobia have a better nitrogenase activity than temperate rhizobia on sainfoin when plants or nodules are exposed to low temperature during short-term incubations. However, further studies are required to ascertain whether this characteristic can improve the yield of sainfoin when the symbiotic system is exposed to low temperatures in greenhouse and field experiments.

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