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# Nitrogen fixation at phyllospheric level in coniferous plants in Italy

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*Key words:* nitrogen fixation, phyllosphere, *Pinus nigra*, *Pseudotsuga menziesii*

**Abstract**

The results of a three year investigation on phyllospheric nitrogen fixation in *Pinus nigra* and *Pseudotsuga menziesii* growing in Central Italy are reported. The highest levels of nitrogen fixation in *Pinus nigra* and *Pseudotsuga menziesii* needles were reached during spring in three year old needles (from 6.1 to 7.6 and from 8.1 to 10.2 nmoles of $N_2$ fixed $h^{-1}$ $g^{-1}$ needles) collected in the center of the canopy, while the lowest values (less than 1.5 and 3.0 nmoles of $N_2$ fixed $h^{-1}$ $g^{-1}$ needles) were detected during summer in young needles collected in the lateral branches (areas more exposed to the light and with low humidity). Microorganisms belonging to the genera Pseudomonas, Bacillus, Achromobacter, Klebsiella and Mycobacterium were isolated from the needles of either *Pinus nigra* or *Pseudotsuga menziesii*.

## Introduction

Nitrogen fixation in the forest ecosystem represents the main nitrogen input for the plants. Most of the nitrogen supplies, for the forest environment, are covered by symbiotic and rhizospheric nitrogen fixation (Bond, 1974; Bond, 1983; Tarrant and Trappe, 1971), but not less important are the nitrogen fixing activities of the lichens and of phyllospheric bacteria (Denison, 1979; Ruinen, 1974).

Ruinen (1965) reported firstly, the occurrence, in tropical forest, of nitrogen fixing microorganisms on leaves, but successive reports by several authors (Balloni *et al.*, 1987, 1988; Granhall and Lindberg, 1978; Jones, 1970, 1976, 1978; Sucoff, 1979; Todd *et al.*, 1978) showed nitrogen fixing activity also on leaves of forest plants growing in temperate zones. The nitrogen fixed in the phyllosphere could be used directly by the phyllospheric microflora, taken up by foliage or released into the soil by litter fall or decay. In Italy, researches on phyllosphere nitrogen fixation were lacking until the eighties and estimations on the amounts of nitrogen fixed in the phyllosphere of forest trees were made on the basis of results obtained from measurement made in Northern and Central Europe, North America and New Zealand.

In order to bring a first contribution to the knowledge of the phyllospheric nitrogen fixation as well as of the microorganisms involved in forest plants living in Italy, a three year investigation on the phyllosphere of *Pinus nigra* and *Pseudotsuga menziesii*, was undertaken.

## Materials and methods

### *Study sites*

The *Pinus nigra* Arn. subsp. *nigricans Host* plantation was located in Monte Calvi (Northern Tuscan) in a 70 ha pinewood (elevation 300 m above sea level).

The soil, derived from calcareous bedrock, is acid on the surface and sub-alkaline in the lower horizon. The total nitrogen content was very low (less than 0.1%).

The Douglas fir (*Pseudotsuga menziesii* (Mirb)

Franco) plantation was situated at Consuma (Appennine mountains south-east of Florence) in a 2300 $m^2$ experimental plot. The soil is an acid brown earth, 80–110 cm in depth.

*Sampling*

*Pinus nigra* and Douglas fir needles were collected seasonally over three years from the top, centre and lateral branches of the canopy of different aged plants.

The needles, removed with sterile forceps and scissor, were placed in clean polythene bags and brought immediately to the laboratory. The needles were separated into age classes (1 and 3 years old).

Nitrogen fixing activity was estimated by the acetylene reduction method (Burris, 1974). Small quantities (1.0 g d.w.) of needle samples were introduced in 70 mL glass vials with serum caps containing 2 mL of sterile distilled water. The gas phase was air containing 12.5% $C_2H_2$ by volume. The vials were incubated at 33°C. The gas phase was sampled after 6 hours. A molar ratio of 3:1 to convert $C_2H_4$ formed into $N_2$ fixed was assumed.

*Isolation, characterization and counting of nitrogen fixing microorganisms*

Nitrogen fixing bacteria were isolated from enrichment cultures prepared according to the method described elsewhere (Balloni *et al.*, 1987), by successive plating on solid Rennie medium (Rennie, 1981).

The strains isolated were identified according to the criteria reported on Bergey's Manual of Systematic Bacteriology (Krieg *et al.*, 1984).

The CFU of nitrogen fixing bacteria per gram (d.w.) of needles was estimated by the MPN method (Hegazi *et al.*, 1979).

## Results and discussion

The seasonal time course of the nitrogenase activity detected in *Pinus nigra* and *Pseudotsuga menziesii's* three year old needles is shown in Fig. 1.

The nitrogenase activity, evaluated by the acetylene reduction test directly on needle samples, was high in spring (6.1 and 8.0 nmoles of $N_2$ fixed $h^{-1}$ $g^{-1}$ respectively for *Pinus nigra* and *Pseudotsuga menziesii*) and lower in summer (1.5 and 3.6 nmoles of $N_2$ fixed $h^{-1}$ $g^{-1}$) while negligible in winter. Moreover, the phyllospheric nitrogen fixation was higher in the oldest needles (more than three years old) collected in the centre of the canopy while the lowest values (less than 1.5 nmoles $N_2$ fixed $h^{-1}$ $g^{-1}$ d.w. needles) were detected in young needles collected in the top and lateral branches of the canopy (Tables 1 and 2).

The number of the nitrogen fixing microorganisms reached the maximum value in spring and in fall (7.0 and 6.0 CFU $\times 10^6$ $g^{-1}$ needles respectively in *Pinus nigra* and *Pseudotsuga menziesii*) and was in accordance with the nitrogenase activity data (Table 3).

The nitrogen fixing microorganisms of *Pinus nigra* and *Pseudotsuga menziesii* phyllosphere were represented by bacteria belonging to the genera Pseudomonas, Klebsiella, Achromobacter, Bacillus and Mycobacterium (Table 4).

From the results obtained during three years of research on the phyllospheric nitrogen fixation

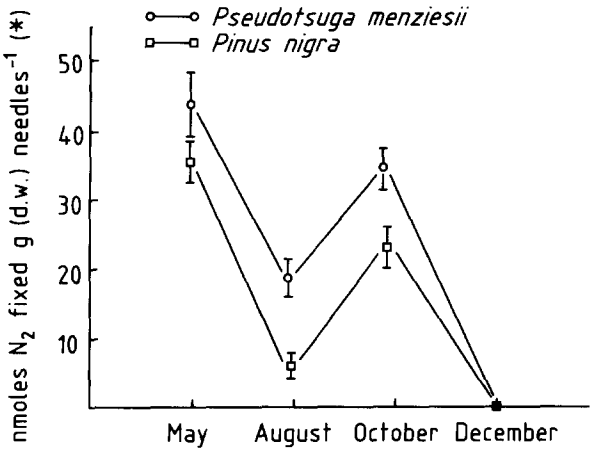


*Fig. 1.* Seasonal time course of nitrogen fixation by *Pinus nigra* and *Pseudotsuga menziesii* three year old needles (Average of samples collected from 10 plants. *Data are means of three experiments each with six replicates ± standard deviation).

*Table 1.* Nitrogenase activity in different aged needles collected in different parts of the canopy (average of samples collected in September from 10 plants)

| Needle's age (years) | Position of needles in canopy | nmoles $N_2$ fixed g (w.d. needles)$^{-1}$ $h^{-1}$ | |
|---|---|---|---|
| | | *Pinus nigra* | *Pseudotsuga menziesii* |
| 1 | | 2.0 | 1.5 |
| 3 | Exterior | 4.0 | 4.2 |
| | Inner | 9.3 | 10.8 |
| | Top | 5.1 | 8.7 |
| | Intermediate | 8.0 | 9.5 |
| | Low | 4.2 | 5.8 |
| Mean value | | 6.1 | 8.0 |

*Table 2.* Nitrogenase activity on *Pseudotsuga menziesii*'s needles collected from plants of different age (average of samples collected from 10 plants)

| Age (years) | nmole $N_2$ fixed g(d.w. needles)$^{-1}$ $h^{-1}$ | | |
|---|---|---|---|
| | Spring (May) | Summer (July) | Autumn (September) |
| 10–15 | 5.8 | 2.5 | 6.4 |
| 25–30 | 10.7 | 3.2 | 7.8 |
| over 50 | 8.5 | 2.0 | 8.0 |

*Table 3.* Seasonal variation of the CFU of nitrogen fixing bacteria in *Pinus nigra* and *Pseudotsuga menziesii*'s needles (average results of 3 year investigation)

| Season | Nitrogen fixing bacteria, CFU $\times 10^6$ $g^{-1}$ | |
|---|---|---|
| | *Pinus nigra* | *Pseudotsuga menziesii* |
| Spring | 7.0 | 5.0 |
| Summer | 0.003 | 0.006 |
| Fall | 4.0 | 6.0 |

*Table 4.* Nitrogen fixing bacteria isolated from *Pinus nigra* and *Pseudotsuga menziesii*'s needles

| Species | Number of strains isolated | |
|---|---|---|
| | *Pinus nigra* | *Pseudotsuga menziesii* |
| *Pseudomonas fluorescens* | 4 | 5 |
| *Bacillus polymyxa* | 1 | 2 |
| *Achromobacter* sp. | 3 | – |
| *Mycobacterium* sp. | 1 | – |
| *Klebsiella aerogenes* | – | 3 |

in *Pinus nigra* and *Pseudotsuga menziesii* it is possible to draw some important points:

a) The highest nitrogenase activity, evaluated by acetylene reduction test directly on needle's samples, without enrichment on nitrogen free medium, was detected in spring (8.1 nmoles of $N_2$ fixed $h^{-1}$ $g^{-1}$) while the lowest was reached in summer (1.5 nmoles of $N_2$ fixed $h^{-1}$ $g^{-1}$).

b) From the phyllospheric nitrogen fixation values obtained, assuming a leaf biomass weight of the entire plant ranging from 3000 to 15000 kg $ha^{-1}$ (Grier *et al.*, 1974; Ovington *et al.*, 1963), it is possible to estimate the amounts of $N_2$ fixed, respectively, by *Pinus nigra* and *Pseudotsuga menziesii* phyllospheres, to be 8 kg and 12 kg $ha^{-1}$ $yr^{-1}$. These estimations, based on the highest values of $C_2H_2$ reduction reached in all the samples of *Pinus nigra* and *Pseudotsuga menziesii*'s needles, are in agreement with those reported by Todd *et al.* (1978) in coniferous plants at temperate zones (Table 5).

c) The nitrogen fixing microorganisms of the *Pinus nigra* and *Pseudotsuga menziesii* phyllosphere were represented by *Pseudomonas fluorescens*, *Bacillus polymyxa*, *Klebsiella aerogenes*, *Achromobacter* sp. and *Mycobacterium* sp. Typical nitrogen fixing soil genera such as Azotobacter and Beijerinckia were not found.

The research is in progress and will be extended to other coniferous stands of central and southern Italy.

*Table 5.* Comparison of nitrogen fixation rates in forest plants

| Forest type | Estimated kg $N_2$ fixed $ha^{-1}$ $yr^{-1}$ | References |
|---|---|---|
| **Coniferous forest** | | |
| Douglas fir (Oregon, USA) | 12 | Todd *et al.*, 1978 |
| Douglas fir (England) | 20 | Jones *et al.*, 1974 |
| Mixed conifers (New Zealand) | 12.5 | Silvester and Bennett, 1973 |
| *Pinus nigra* (Italy) | 9 | This paper |
| *Pseudotsuga menziesii* (Italy) | 12 | This paper |
| **Broadleaf forest** | | |
| Tropical rain forest (Puerto Rico) | 88 | Edmisten, 1970 |
| Tropical rain forest (Costa Rica) | 1–8 | Forman, 1975 |
| Mixed hardwood (New Hampshire, USA) | 14 | Todd *et al.*, 1978 |
| Mixed hardwood (North Carolina, USA) | 12 | Todd *et al.*, 1978 |

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