Pak.J.Bot., 26(2): 241-246,1994.

SEASONAL FLUCTUATIONS IN NITROGEN-FIXING ABILITY (C_2H_2 REDUCTION) AND HYDROGEN UPTAKE BY ROOT NODULES OF *CORIARIA NEPALENSIS* AND *DATISCA CANNABINA*

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Abstract

A study was made on the seasonal fluctuations in N_2-fixing (C_2H_2 reduction) activity and uptake hydrogenase activity of the root nodules of *Coriaria nepalensis* and *Datisca cannabina*. Nitrogenase activity of the nodules of both plants showed biphasic curves with peaks in spring and late summer. Acetylene reduction by the root nodules of *Coriaria nepalensis* was highest (16.4 μ mol. $C_2H_4.g^{-1}$ fresh nodule wt. h^{-1}) during July while the peak acetylene reduction activity (14.8 μ mol. $C_2H_4.g^{-1}$ fresh nodule wt. h^{-1}) of *Datisca cannabina* nodules was determined in April. Less than 15% of the enzyme activity was retained by the nodules in winter. The nodules of both plant species consumed hydrogen on incubation with a gas mixture containing H_2, indicating an uptake hydrogenase activity. The H_2 uptake by the excised nodules of both *Coriaria* and *Datisca* was highest (2.16 and 1.95 μ mol. H_2 consumed g^{-1} fresh nodule wt. h^{-1}, respectively) in May. The presence of an uptake hydrogenase was confirmed in nodule homogenates with phenazine metasulphate as an artificial electron acceptor. Purified vesicle cluster suspensions (20 μm residue) showed highest hydrogenase activity, indicating that the enzyme is associated with the endophyte.

Introduction

Nitrogen-fixing shrubs *Coriaria nepalensis* and *Datisca cannabina* have great potential for utilization as fast growing pioneers in the hilly areas affected by landslides and erosion (Chaudhary *et al.*, 1985). Like other N_2-fixing microorganisms, the actinomycetous endophyte (*Frankia*) present in the root nodules of actinorhizal plants can reduce atmospheric nitrogen with the help of the nitrogenase system. During reduction of N_2 by microorganisms, considerable amount of energy is lost in the simultaneous process of reduction of protons to H_2 by the nitrogenase (Arp, 1990; Evans *et al.*, 1987). Several nitrogen fixers posses an uptake hydrogenase which recycles this H_2, and regenerates energy and reducing power for reutilization by nitrogenase (Evans *et al.*, 1987). It has been suggested that this reaction also provides a means of protection of nitrogenase from oxygen (Dixon, 1972).

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Uptake hydrogenase activity has been reported in the *Frankia* induced nitrogen fixing root nodules of non-legumes (Huss-Danell, 1990). Vesicle cluster suspensions, which contain endophytic tissue released from host cells, have been successfully used to study various physiological processes such as nitrogenase activity, uptake hydrogenase activity and other metabolic processes of the endophyte (Huss-Danell, 1990).

In this paper seasonal variations in nitrogen-fixing ability (C_2H_2 reduction) and hydrogen uptake by the root nodules of *Coriaria nepalensis* and *Datisca cannabina* is discussed. Furthermore, hydrogen uptake by different fractions of the nodule homogenates using artificial electron acceptors is described.

Materials and Methods

Plant growth: To avoid extensive travelling for collection of fresh nodules from the plants growing in their natural habitats, seedlings of *Coriaria nepalensis* and *Datisca cannabina* were raised in the nursery of Quaid-e-Azam University Islamabad, Pakistan. Temperatures at Islamabad are considerably higher as compared to the natural habitats of *Coriaria* and *Datisca*. This is especially true for Swat where *Datisca* plants remain covered with snow for a considerable period of time during winter. *Coriaria* nodules used for inoculum were collected from Murree while *Datisca* nodules were collected from Swat. The seedlings were grown in sterile sand and inoculated at the age of 8 weeks with crushed nodule suspension. N-free Hoagland nutrient solution (half strength) was added once a week. Nodules were collected from the plants for one year (March 1986-Feb. 1987) and used for measuring acetylene reduction activity and uptake hydrogenase activity.

Acetylene reduction: Nitrogenase activity was measured by incubating nodulated roots in air-tight plastic bottles (500 ml) with air and acetylene (10 %) at ambient air temperature. One ml gas samples from these plastic bottles were transferred after every 15 minutes to vacutainer tubes (5.6 ml). The gas samples were stored in these tubes and analyzed for C_2H_4 production with FID system of gas chromatgraph (Hitachi Model 163). Carrier gas (N_2) was 40 ml. min^{-1}. Porapak R, packed loop steel column, 1.5 m long and 3 mm in ID was used for separation.

H_2-uptake by excised root nodules: H_2-uptake by the nodules was measured on a gas chromatograph as described earlier (Mirza *et al.*, 1987).

Preparation of nodule homogenate for H_2 uptake: The nodule homogenates, from green house collected material, were prepared as described by Roelofsen & Akkermans (1979). H_2-uptake by the homogenates was measured on a gas chromatograph in the presence of different electron acceptors.

Results

Root nodules of *Coriaria* and *Datisca* collected from the plants growing under similar conditions showed nitrogenase activities of the same order of magnitude. Acetylene reduction activity recorded during different seasons varied considerably and showed peak enzyme activity twice a year (Fig. 1). The activity was low during winter and increased progressively during spring. The acetylene reduction activity decreased

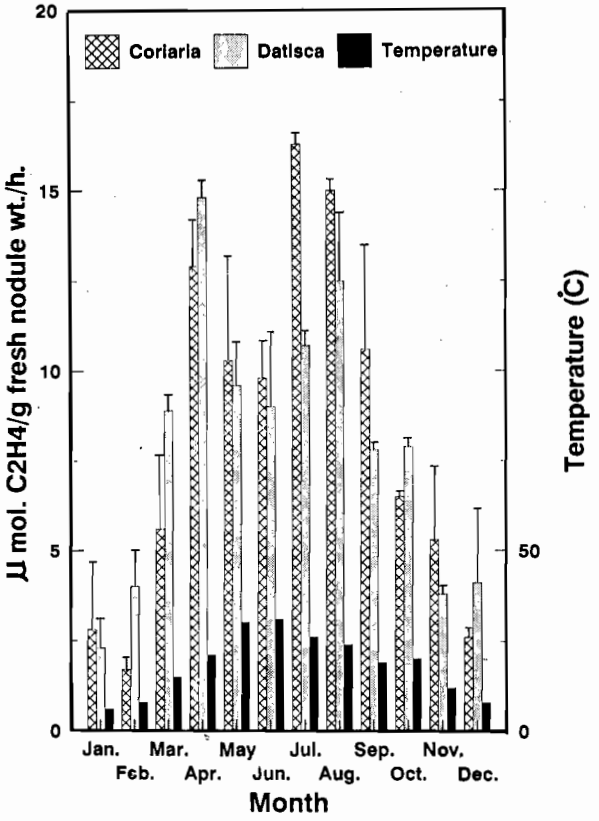

Fig.1. Seasonal fluctuations in nitrogen-fixing ability (C_2H_2 reduction) by root nodules of *Coriaria nepalensis* and *Datisca cannabina*.

during hottest months of May and June and recovered again during July after monsoon rains. The highest acetylene reduction activity (16.4 μ mol. C_2H_4. g^{-1} fresh nodule wt. h^{-1}) by *Coriaria* nodules was determined in July. In *Datisca* root nodules, peak acetylene reduction activity (14.8 μ mol. C_2H_4. g^{-1} fresh nodule wt. h^{-1}) was recorded in April.

Root nodules of *Coriaria* and *Datisca* did not evolve any detectable amount of hydrogen throughout the year. However, the nodules consumed hydrogen on incubation with a gas mixture (Fig. 2). For both *Coriaria* and *Datisca* nodules, the highest H_2 uptake activity (2.16 and 1.95 μ mol. H_2 consumed. g^{-1} fresh nodule wt.h^{-1}, respectively) was determined in May. The uptake hydrogenase activity was detected in both fractions of the nodule homogenates. The activity was higher in 20 μm residual fraction than 20 μm filtrate (Table 1). The activity was absent in the supernatant obtained by centrifugation of 20 μm filtrate fraction. Intact nodules of *Coriaria* and *Datisca* used for preparation of homogenates consumed H_2 at the rate of 1.9 and 1.6 μ mol. H_2. g^{-1} fresh nodule wt. h^{-1}, respectively. The activity of uptake hydrogenase

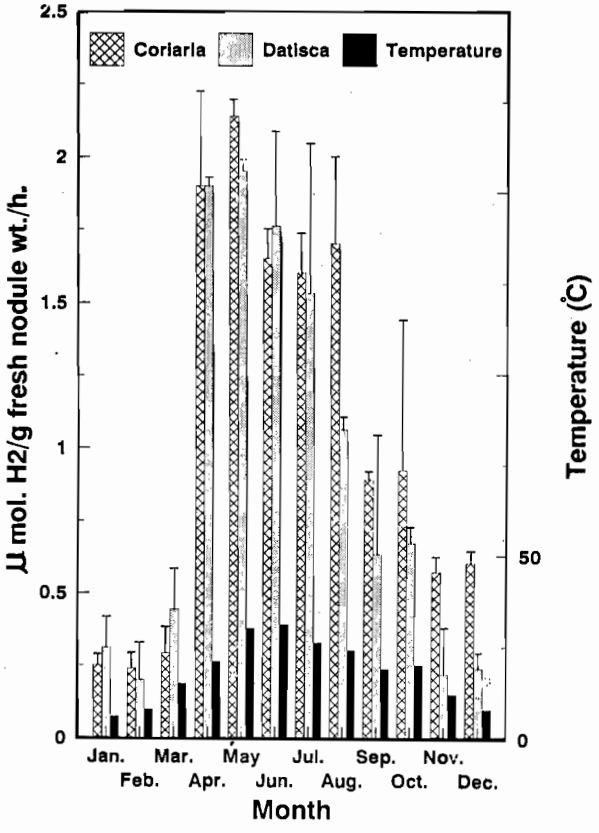

Fig.2. Seasonal fluctuations in uptake hydrogenase activity by root nodules of *Coriaria nepalensis* and *Datisca cannabina*.

measured in case of nodule homogenate (20 μm residue + filtrate) of *Coriaria* and *Datisca* in the presence of phenazine metasulfate were 1.3 and 0.7 μ mol. H_2 g^{-1} fresh nodule wt. h^{-1}., respectively.

Under the microscope, the 20 μm residue mainly consisted of vesicle clusters of the endophyte. The 20 μm filtrate contained only broken parts of host cells and of the endophyte.

Discussion

The rate of acetylene reduction by nodules of *Coriaria nepalensis* and *Datisca cannabina* are comparable to those already reported for actinorhizal plants of Pakistan (Chaudhary *et al.*, 1985). During winter the plants survive in leafless dormant state and the enzyme activity is low due to low temperatures and limited supply of photosynthates required for the energy intensive process of nitrogen fixation. With the emergence of leaves in early spring, the activity increased and reached its peak in April. After relatively low enzyme activity in hottest months of summer when temperatures are

Table 1. H_2 uptake by intact nodules and nodule homogenates of *Coriaria nepalensis* and *Datisca cannabina* .

Nodule material used	Coriaria nepalensis	Datisca cannabina
	*μmol. H_2 consumed. g^{-1} fresh nodule $wt.h^{-1}$	*μmol. H_2 consumed. g^{-1} fresh nodule $wt.h^{-1}$
Intact nodules	1.9	1.6
Nodule homogenate	1.3	0.7
20 μm residue (vesicle cluster suspension)	1.0	0.5
20 μm filtrate pellet	0.3	0.2
20 μm filtrate supernatant	0	0

*Each value represents average of three determinations

considerably higher than those of the natural habitats, the activity recovered again during rainy season (July-August). This again is correlated with the vigorous vegetative growth of plants under the given growing conditions. In an early study using nodules of *Alnus glutinosa* and *Alnus rubra*, it was observed that the period of maximum nitrogenase activity coincides with that of maximum growth (Wheeler *et al.*, 1981).

Like several other actinorhizal nodules an uptake hydrogenase is also present in nodules of *Coriaria nepalensis* and *Datisca cannabina* (Hafeez *et al.*, 1984; Mirza *et al.*, 1987). This indicates presence of a more efficient nitrogen fixing system as has been reported for *Rhizobium*-nodulated plants (Arp, 1990). The nodules always consumed H_2 from the gas phase while no net H_2 evolution was detected during any season. Using nodules collected from natural habitat, H_2 consumption by *Coriaria* nodules was detected throughout the year (Mirza *et al.*, 1987). It has been reported previously (Hafeez *et al.*, 1984) that *Datisca* nodules evolve H_2 in summer. However, this H_2 evolution was suggested to be due to the deterioration of the nodule material used for the assay during transportation from a long distance. The results of the present study using fresh nodules of *Coriaria* and *Datisca* nodules differ from that of *Alnus* where H_2 evolution have been detected during autumn (Roelofsen & Akkermans, 1979). High uptake hydrogenase activity of both *Coriaria* and *Datisca* nodules was detected during early summer when conditions for vegetative growth of the host plant were favourable or possibly due to stimulation by the increase in H_2 production by nitrogenase.

Hydrogenase activity is present in vesicle cluster suspension as well as filtrate fractions of the nodule homogenates. High enzyme activity in 20 μm residue is obviously due to the presence of intact vesicle clusters of the endophyte. The activity in the filtrate is also particle bound as exhibited in the fraction while activity was absent in the supernatant. The fact that uptake hydrogenase activity of the nodule homogenate (20 μm residue + filtrate) is less than that of the intact nodules may be due to partial inactivation of the enzyme or alternately all of the vesicle clusters may not be released from the host cells during homogenization. The results obtained with *Datisca* nodule homogenates in the present study are comparable to those reported previously (Hafeez *et al.*, 1984).

The results with nodule homogenates of *Coriaria* and *Datisca* indicate that the uptake hydrogenase is associated with the endophyte. Use of modern techniques e.g., *in situ* hybridization and isolation of the endophyte will greatly facilitate localization of the enzyme in hyphae or vesicles of the endophyte.

Present studies with *Coriaria* and *Datisca* indicate that maximum nitrogenase activity of the nodules coincides with the period of vigorous plant growth. This suggests that during fast growth period increased nitrogenous demands of the host plant are met with the elevated nitrogen fixation rates by the endophyte.

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(Received for Publication 6 June 1994)